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The Role of Adenosine in Airway Epithelial Ion Channel Regulation

By

Artur J. Szkotak

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Physiology

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UNIVERSITY OF ALBERTA

Faculty of Graduate Studies and Research

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ABSTRACT

As the layer of tissue exposed directly to the microbe rich air, the airway epithelium is largely responsible for preventing infection. It is believed that the epithelium achieves this by mediating mucociliary clearance, secreting antibiotic factors, and regulating airway surface liquid pH; all of which are dependent on salt and therefore ion transport. Thus, when ion transport becomes compromised, infection results. The best example of this is Cystic Fibrosis (CF), a lethal disease characterized by progressive lung infection, caused by mutations in the CFTR Cl^- channel.

This study has focused on the regulation of airway epithelial ion transport by the endogenous autocrine/paracrine mediator – adenosine, in three model cell systems: the A549 cell line, the Calu-3 cell line, and primary cultures of normal human bronchial epithelium (NHBE). The regulatory actions of adenosine are mediated by four distinct membrane receptors. We have used complementary molecular and functional techniques to show that A_1 receptors are responsible for ion channel regulation in A549 cells, while A_{2A} and A_{2B} receptors are involved in Calu-3 and NHBE cells.

Further study revealed that the concentration of adenosine in the vicinity of these receptors is controlled by at least three factors: equilibrative nucleoside transporters (ENTs), adenosine kinase, and 5'-nucleotidase. ENT1, mediates the rate-limiting step in adenosine signal termination, such that inhibition of this transporter stimulates adenosine-dependent ion transport. Adenosine kinase and 5'-nucleotidase were found to be important in the metabolism and production of adenosine, respectively.

The ion channel targets of adenosine signaling were also investigated. In A549 cells, adenosine regulates the intermediate conductance Ca^{2+}-dependent K^+ channel. In contrast, in Calu-3 and NHBE cells, cAMP-dependent channels are targeted, in particular CFTR and an Outwardly Rectifying Cl^- Channel. The basolateral localization of the latter was further characterized, and a novel role for this channel was proposed in anion secretion that may explain how preferential HCO_3^- secretion occurs.

Thus, we have characterized the factors involved in adenosine-dependent ion transport. Furthermore, we have proposed pharmacological agents that target these factors, some of which are already approved for clinical use, which may be of benefit in the treatment of CF.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
AKAP	A-Kinase Anchoring Protein
AQP	Aquaporin
ASL	Airway Surface Liquid
A_X	Adenosine Receptor; X denotes the subtype (1, 2A, 2B or 3)
BK	Big Conductance Ca^{2+} Dependent K^+ Channel
$[Ca^{2+}]_i$	Intracellular Ca^{2+} Concentration
CaMK II	Calmodulin Kinase II
cib	Concentrative, Insensitive to NBMPR, Broadly Selective
cif	Concentrative, Insensitive to NBMPR, Formycin B Selective
cit	Concentrative, Insensitive to NBMPR, Thymidine Selective
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CGS-21680	2-p-(2-Carboxyethyl)phenethylamino-5'-(N-ethylcarboxamido)adenosine
ClC	Voltage Gated Cl^- Channel
CLCA	Ca^{2+} Dependent Cl^- Channel
cs	Concentrative, Sensitive to NBMPR
csg	Concentrative, Sensitive to NBMPR, Guanosine Selective
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-Disulfonic Acid

DPC	Diphenylamine-2-Carboxylate
DPCPX	1-3-Dipropyl-8-Cyclopentylxanthine
EBIO	1-Ethyl-2-Benzimidazolone
EBP50	Ezrin Binding Phosphoprotein of 50 kDa
ei	Equilibrative-Insensitive
ENaC	Epithelial Na^+ Channel
es	Equilibrative-Sensitive
G-protein	Guanosine Triphosphate Binding protein
GABA	γ-Amino-Butyric Acid
GPCR	G-Protein Coupled Receptor
hCNT	Human Concentrative Nucleoside Transporter
hENT	Human Equilibrative Nucleoside Transporter
$I_{Cl,swell}$	Swelling-Activated Cl^- Channel
IK	Intermediate Conductance Ca^{2+} Dependent K^+ Channel
I_{sc}	Short-Circuit Current
K_{ir}	Inwardly Rectifying K^+ Channels
K_m	Michaelis-Menten Constant
K_v	Voltage Dependent K^+ Channels
NBC	Na^+-HCO_3^- Cotransporter
NBD	Nucleotide Binding Domain
NBMPR	Nitrobenzylmercaptopurine Ribonucleoside
NBTI	Nitrobenzylthioinosine
NECA	5'-(N-Ethylcarboxamido)adenosine

NHBE	Normal Human Bronchial Epithelium
NKCC	Na^+-K^+-$2Cl^-$ Cotransporter
NPPB	5-Nitro-2-(3-Phenylropylamino)benzoic Acid
NSC	Non-Selective Cation Channel
ORCC	Outwardly Rectifying Chloride Channel
P_1	Purinergic Receptor Subfamily 1 (Adenosine Receptors)
P_2	Purinergic Receptor Subfamily 2 (Nucleotide Receptors)
P Domain	Pore Domain
PKA	Protein Kinase A
PKC	Protein Kinase C
R Domain	Regulatory Domain
R_T	Transepithelial Resistance
RVD	Regulatory Volume Decrease
SK	Small Conductance Ca^{2+} Dependent K^+ Channel
SUR	Sulphonylurea Receptor
TMD	Transmembrane Domain
TMS	Transmembrane Segment
VSOAC	Volume-Stimulated Organic Osmolyte/Anion Channels
UTP	Uridine 5'-Triphosphate
V_{max}	Maximal (Reaction) Velocity
V_T	Transepithelial Potential Difference

CHAPTER 1

INTRODUCTION

1. Airway epithelial salt transport:

1.1 The airway epithelium:

Despite being continuous with the external environment, healthy airways are sterile below the first bronchial division (225). This feat is achieved by the epithelium, a continuous single layer of cells that lines the entire surface of the airways. The proximal epithelium of the trachea and bronchi is pseudostratified, ciliated, and interspersed with mucous producing goblet cells. The epithelium invaginates into numerous specialized submucosal glands, which increase the surface area available for the production of secretions. These glands contain serous and mucous cells, which are the main source of their respective secretions. Mucous cells, secrete the majority of glycoproteins (mucins) and proteoglycans that give mucous its gel-like consistency, while serous cells are responsible for the secretion of watery electrolytes and antibiotic factors. The distal airway epithelium of the bronchioles is cuboidal and less frequently ciliated. In this region of the airway there is a lack of submucosal glands and goblet cells; however, interspersed serous cells are present. Some of these are Clara cells, a clearly distinguishable non-ciliated cell type with an arching apical membrane that projects into the airway lumen. These cells in particular are thought to be important for producing non-mucous secretions, including watery electrolyte (192), and antimicrobial proteins (160).

The airways terminate at the alveoli, which are also covered in epithelium. Two types of non-ciliated cells, designated Type I and Type II pneumocytes, cover 90% and 10% of

the alveolar surface, respectively. The purpose of the type I cells is to provide the least amount of resistance to gas exchange possible. They achieve this by being exceedingly thin, approximately 0.2 μm (221), compared to their 80 μm diameter (44). Type II pneumocytes are cuboidal and much more metabolically active than the type I cells. Their primary role in the alveolus is to secrete surfactant and antimicrobial factors as well as to participate in fluid and electrolyte transport.

1.2 Salt in the airway:

The most abundant, and therefore the most important salts in physiological fluids are comprised of Na^+, K^+, Ca^{2+}, Cl^- and HCO_3^- ions. The concentrations of these ions in the airways vary between the plasma, the intracellular fluid and Airway Surface Liquid (ASL) secreted into the lumen. Under normal conditions the plasma can be considered as an essentially infinite reservoir of watery electrolytes, whose concentrations are maintained within a narrow range (in mM): Na^+, 145; K^+, 4; Ca^{2+}, 2.5; Cl^-, 103; and HCO_3^-, 24. Thus, airway epithelial cells use this reservoir as a source or sink of fluid and electrolyte. The actual amounts of these ions and water found intracellularly and in the ASL varies greatly, and can be altered through regulated processes.

Airway epithelial cells maintain and regulate their negative membrane potential by altering intracellular ion concentrations and membrane permeabilities. The Goldman-Hodgkin-Katz equation (1.1) relates these factors mathematically:

$$E = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i + P_{HCO_3}[HCO_3]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o + P_{HCO_3}[HCO_3]_o} \qquad (1.1)$$

Where E is the membrane potential, R is the gas constant (8.315 $J \cdot K^{-1} \cdot mol^{-1}$), T is the temperature (K), F is the Faraday constant (9.648 x 10^4 C/mol), P_X is the permeability of

the membrane to ion *X*, [*X*] is the concentration of ion *X*, and *i/o* refer to intra-/extra-cellular. Ca^{2+} has been omitted from equation 1.1 because its concentrations are so small that it will not contribute significantly to membrane potential. Membrane potential depends primarily on Na^+ and K^+ gradients established and maintained by the Na^+/K^+-ATPase, which are outwardly directed for K^+ ($K_o/K_i < 1$) and inwardly directed for Na^+ ($Na_o/Na_i > 1$). Because $P_K > P_{Na}$ in non-excitable cells, K_o/K_i dominates the equation and since the natural log of a value less than 1 is negative, so is membrane potential. Furthermore, the greater P_K becomes, due to the opening of K^+ channels, the more negative *E* becomes. Active transport of Cl^- and HCO_3^- is much less significant, then that of the cations, such that these anions normally equilibrate near the membrane potential. In other words, while Na^+ and K^+ concentrations, established by the Na^+/K^+-ATPase, determine membrane potential, Cl^- and HCO_3^- concentrations are determined by it.

One of the lingering problems in airway epithelial research is the question of ion concentrations in the ASL. Accurate measurements have been difficult because of the very small volumes involved and due to the proximity of the fluid to the epithelium. Standard techniques such as the filter paper method, which relies on the absorption of a particular secretion onto filter paper, produces unreliable results when used in the airway because it stimulates epithelial secretion. Furthermore, due to the very thin nature of the ASL layer (10-50 μm) (6,39,217), when compared to the relatively large surface area of the airway, it is quite likely that its depth and composition change with each breath as water evaporates. Thus, external factors such as respiratory rate, and the quality of the air respired are likely to have a profound influence on the composition of the ASL. Numerous recent studies using new instrumentation and techniques such as miniature

solid state electrodes (25), *in vivo* microdialysis (82), fluorescent probes (106), and radiotracers (223,232) have supported both isotonic (25,82,106) and hypotonic, ~50 mM NaCl (223,232), ASL composition as compared to plasma. Thus, an accurate portrayal of ion concentrations in the ASL remains elusive.

1.3 The purpose of salt in the airway:

The principal task of the epithelium is to defend the lung from inhaled pathogens and particulates. It achieves this task through at least three salt-dependent mechanisms: the mucociliary escalator, antibiotic factors and the regulation of ASL pH (39,162).

As its name implies, the mucociliary system is composed of two parts – mucous and cilia. The viscous mucous, coats the epithelial surface and traps inhaled pathogens and particulates. The beating of epithelial cilia functions to move the mucous from the airways towards the pharynx, where it can be either swallowed or expectorated. The mucous coat is actually only the outermost stratum of a more complex layer of fluid – the ASL (162). The ASL is divided into the aforementioned outer mucous layer, also known as the gel layer and the inner periciliary or sol layer. The latter is a low viscosity medium composed mostly of water and electrolytes. The depth of the periciliary layer and the length of the cilia it bathes are normally equal (~6 μm), such that only the tips of the cilia contact the mucous layer (162). This is a very important relationship because it allows for efficient beating in a low viscosity medium, while at the same time providing for contact between the cilia and the mucous layer. This model implies that if the periciliary layer were to be too thin the high viscosity mucous layer would hinder ciliary beating, while if it were too thick the cilia would lose contact with the mucous layer. However, in practice

it seems that an excessively deep periciliary fluid does not occur because the mucous layer can act as a sponge, soaking up surplus fluid (20). Thus, it is depletion of the periciliary fluid that impairs mucociliary clearance. Furthermore, the periciliary fluid acts as a lubricant between the epithelial cell surface mucins and those within the mucous layer, preventing their entanglement and thereby facilitating cough clearance (20). Clearly, maintenance of the depth of the periciliary fluid, determined by its degree of hydration, is very important for the proper functioning of clearance mechanisms. However, because water cannot be actively transported, the epithelium must manipulate salt concentrations in order establish the driving force for fluid movements.

There is also evidence that salt participates in the epithelial regulation of anti-microbial defense. It is known that ASL is more than simply salt and water. Proteases/antiproteases, oxidants/antioxidants, and antibiotics all co-exist in the ASL and work together to impede infection while also preventing collateral damage to the lung. Smith et al. (194) showed that high salt concentrations in the ASL inactivate factors with bactericidal activity produced by the epithelium. Further studies revealed that ionic strength, in particular, was responsible for these effects, since non-ionic substances did not affect bactericidal activity (213). These findings were soon followed by the identification of anti-microbial factors, termed β-Defensin-1 and -2, secreted by airway epithelia that are sensitive to inhibition by NaCl concentrations greater than 50 mM (11,77). Furthermore, it was found that high salt concentrations reduced the synergy between the major antimicrobial factors – lysozyme and lactoferrin (193). Therefore, salt may also be involved in airway defense by determining the activity of anti-microbial factors.

The importance of the airway epithelium, which can secrete HCO_3^- salts, in maintaining ASL pH is only beginning to be recognized (166). It has been shown that significant deviations in ASL pH, from its normal value of 6.5-7.0 (105,106,124), has several adverse effects. When the ASL is acidic, protonation of the sulphated and sialated carbohydrate sidechains of mucins not only reduces the hydration state of these molecules but also diminishes electrostatic repulsions between neighbours, resulting in increased mucous viscosity and therefore decreased clearance. ASL acidification also impedes effective immune function by reducing the ability of leukocytes to combat infection while concomitantly increasing the amount of collateral damage done to surrounding tissue (1,191,215). Furthermore, ciliary beat frequency is decreased when the ASL is acidic, presumably resulting in reduced efficiency of the mucociliary escalator (38). As the most important buffer in physiological solutions, the secretion of HCO_3^- must be central in the maintenance of ASL pH.

1.4 Mechanisms of salt transport:

The hydration, salt composition, and pH of the ASL is regulated and maintained by the airway epithelium. While ions can be transported directly, vectorial water transport can only be achieved indirectly by establishing an osmotic driving force. This is accomplished through the active transport of salts, which establish osmotic gradients that pull water in the appropriate direction.

Native epithelial cells are polarized into apical (lumen facing) and basolateral (blood facing) membranes. These membranes contain distinct compliments of transport proteins that are prevented from mixing by tight junctions and through associations with

cytoskeletal elements (57,93). This organization allows for vectorial ion transport. However, exactly how this occurs is a matter of debate, as two, often contradictory, models of airway epithelial ion transport exist. The existence of the isotonic ASL and hypotonic ASL models reflects the uncertainty in the ion concentrations present in this fluid layer (see section 1.2).

The isotonic model of Na^+-absorption and anion secretion, depicted in Figure 1.1A, is based largely on Ussing chamber experiments, performed on epithelial monolayers clamped at a transepithelial potential of zero by an external amplifier. These so called short-circuit current (I_{sc}) measurements have been used extensively to describe ion transport (52,162,195,224). The Na^+/K^+-ATPase and K^+ channels, which together are responsible for maintaining membrane potential, are localized basolaterally. Cl^- is actively transported into the cell via basolateral Na^+-K^+-$2Cl^-$ cotransporters (NKCC) and may exit via apical membrane anion channels. HCO_3^- is actively transported into the cell through basolateral Na^+-HCO_3^- cotransporters (NBC), or can be produced inside the cell by the carbonic anhydrase catalyzed reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Intracellular HCO_3^- then exits the cell, across the apical membrane, via the same channels utilized by Cl^-. Na^+ is transported into the cell, down its concentration gradient, through the apical membrane via the Epithelial Na^+ Channel (ENaC), and is then pumped out across the basolateral membrane by the Na^+/K^+-ATPase. Depending on which process (anion secretion or Na^+-absorption) predominates, the appropriate counter ion will move paracellularly in order to minimize charge separation. In other words, if anion secretion predominates, Na^+ will travel through the tight junctions into the ASL in order to

minimize charge separation, and net NaCl secretion results. Small changes in osmotic gradients, resulting from net NaCl secretion or absorption, cause water fluxes both paracellularly and transcellularly resulting in the maintenanance of isotonic ASL. While the paracellular pathway is known to be involved in water flux, it has only recently been established that transcellular pathways can also contribute. In particular, the role of Aquaporins (AQP), a family of transmembrane proteins that act as water channels, in transcellular water flux is beginning to be recognized. For example, it has been shown that AQP5 knockout mice display significantly reduced submucosal gland fluid secretion (18). The main criticism of the isotonic ASL model is that airway epithelia must operate *in vivo* without the benefit of a voltage clamp amplifier, and consequently do not normally function under short-circuit conditions.

The hypotonic ASL model of airway epithelial ion transport has been proposed more recently (216,232). This model, depicted in Figure 1.1B, is based on Ussing chamber experiments performed under open-circuit conditions in which the epithelium is in equilibrium with no net current flow, thus necessitating measurements of the spontaneous transepithelial potential difference (V_T). Studies under these conditions suggest that Na^+-absorption, through apical ENaC and the basolateral Na^+/K^+-ATPase, creates an electrochemical gradient that favours transcellular Cl^- absorption. In this model Cl^- enters and exits the cell through apical and basolateral anion channels, respectively, thereby neutralizing the charge separation created by Na^+-absorption. Thus, according to this model, transcellular anion secretion does not occur physiologically and is an artifact observed under I_{sc} conditions. Furthermore, it is argued that most I_{sc} experiments are performed in the presence of the ENaC inhibitor – amiloride, which leads to

hyperpolarization of the apical membrane and artificially induces anion secretion. Because transcellular anion secretion does not occur, it is assumed that ASL is produced as a paracellular isotonic transudate, which is then made hypotonic by active epithelial NaCl absorption. One of the great strengths of this model is that if applied to sweat duct epithelium it explains how mutations of the apical Cl^- channel, CFTR, result in abnormally elevated sweat [NaCl], a characteristic of the illness cystic fibrosis, which is caused by just such a mutation.

The main criticism of the hypotonic ASL model is that airway epithelia, unlike sweat duct epithelia, have a low transepithelial resistance (R_T) that is unlikely to sustain a significant osmotic gradient (67,141). Furthermore, the existance of aquaporins suggests that water permeability is even greater than that suggested by measurements of R_T. Even the original authors that proposed this model recognized that the osmotic gradient is too steep to be maintained (232). Thus, they suggested that this might be explained if capillary forces or non-ionic osmolytes assist in its maintainanence. However, subsequent experimentation failed to detect any significant contribution from either of these factors (19,141).

Wine (225) suggests that the differences between the two models can be explained by looking at differences in paracellular permeability. The R_T of monolayers used to support the isotonic ASL hypothesis, >300 $\Omega \cdot cm^{-2}$ (232), were less than those used to support the hypotonic ASL hypothesis, >800 $\Omega \cdot cm^{-2}$ (141). The isotonic ASL hypothesis relies on significant paracellular permeability to exist in order for counter ions to be able to travel through tight junctions to neutralize charge separation and therefore allow for continued secretion/absorption. Alternatively, the hypotonic ASL model relies on high tight

junction resistance in order to maintain hypotonicity. Furthermore, while both studies were carried out on primary cultures of proximal airway epithelium, differences in culture methods could account for the divergent results. Studies by Zabner *et al.* (232) focused on producing epithelial layers with the highest possible R_T, while studies by Matsui *et al.* (141) focused on ciliary development as a marker of differentiation. Whatever the case, these two models of airway epithelial ion transport have polarized the scientific community.

1.5 Deranged airway epithelial salt transport:

Cystic Fibrosis (CF) is the most common lethal genetic disease of Caucasians with an incidence of 1 in 2000 live births. The average life expectancy of individuals afflicted with CF and born after 1990 is 40 years of age (103). Transmission is consistent with an autosomal recessive single gene mutation. The illness is characterized by the production of thick epithelial secretions that lead to plugging and often progressive destruction of hollow organs including the pancreas, the lung, the liver bile ducts, the intestines, sweat ducts and parts of the reproductive tract such as the vas deferens. With the advent of pancreatic enzyme replacement therapy, >95% of CF deaths now occur as a result lung disease (67).

The Cl^- impermeability defect in individuals with CF was discovered by Quinton in 1983 (165) and was followed by the cloning and identification of CFTR as the mutated channel in the late 1980s (13,115,173). Since that time research has been focused on developing a model of the pathogenesis of CF in the hopes that it would suggest ways in which the disease can be treated. However, this has proven to be a difficult task as two

theories of Cl^- transport, the isotonic and hypotonic ASL models, have been proposed. The isotonic ASL hypothesis proposes that CFTR mutations inhibit electrogenic transcellular Cl^- secretion, thereby inhibiting concomitant fluid secretion. This hypothesis also proposes that CFTR normally inhibits ENaC activity, such that its absence results in overactive Na^+- and fluid absorption. Together, this causes periciliary fluid depletion and inhibition of both mucociliary and cough clearance mechanisms, resulting in stasis of secretions and bacterial colonization typical of CF (for review see 20). The hypotonic ASL hypothesis proposes that CFTR mutations inhibit transcellular Cl^- absorption, causing the normally hypotonic ASL to become isotonic, which inhibits the activity of endogenous antimicrobial factors, promoting the bacterial colonization typical of CF (for review see 214). Thus, the hypotonic model proposes that in CF, infection precedes the production of abnormally thick mucous, while the isotonic model proposes that abnormally thick mucous precedes infection. The finding that CF neonates have airway mucus plugs, prior to the onset of infection or inflammation (235) strongly argues in favour of the isotonic model. Furthermore, the hypotonic model predicts that ENaC mutations should result in a disease phenotype similar to, or worse than that caused by CFTR mutation. However, pseudohypoaldosteronism is just such an illness, which results in the production of copious amounts of ASL with none of the infections characteristic of CF (116).

Still a third theory suggests that it is not the lack of Cl^-, but rather the lack of HCO_3^- transport that leads to clinical illness in CF individuals (166). It has been shown that in the pancreas the severity of CF illness correlates with the amount of HCO_3^- transport, but has little correlation with the degree of Cl^- transport (34). Thus, individuals with severe

pancreatic CF phenotype may display significant epithelial Cl^- transport but usually display little or no HCO_3^- transport (34). In the airway, it has been shown that acidic ASL increases mucous viscosity, impairs immune function and paralyzes cilia (see section 1.3), all of which could be involved in the pathogenesis of CF. However, the mechanisms by which HCO_3^- is secreted are ill defined. Furthermore, many epithelial systems, including some from the airway, have been shown to secrete HCO_3^- preferentially over Cl^-. However, how this occurs has not been explained in most cases (47,128,158,197). The fact that $[Cl^-]$ is generally greater than $[HCO_3^-]$ in most physiological fluids, and that CFTR has been estimated to be 4 to 10 times more permeable to Cl^- (99,130), makes preferential HCO_3^- secretion a paradox. One tissue in which the mechanism for preferential HCO_3^- secretion is beginning to be understood is the pancreatic duct epithelium, which appears to secrete Cl^- through apical CFTR channels and then exchange it for HCO_3^- via an apical anion exchanger (197). However, this mechanism cannot account for preferential HCO_3^- secretion in most other tissues, including the airway, which do not display apical anion exchange activity (39,47,132,158). Furthermore, it has been shown that HCO_3^- is likely to be transported directly by CFTR in the airway epithelium (99). Thus, preferential HCO_3^- secretion by epithelia of the airway and many other tissues still requires explanation, but may be important in developing a complete model of CF pathogenesis.

The pathology of other illnesses, particularly those characterized by lung inflammation, such as asthma and chronic bronchitis, may also involve altered ion and fluid transport. Most research into these conditions has focused on increased mucous production as it is well known that submucosal gland hypertrophy and conversion of

serous cells to mucous cells is a major pathological finding. However, the involvement of fluid and electrolytes in this process is beginning to be recognized. It has been shown that inflammatory changes in the circulatory system result in elevated hydrostatic pressure in the submucosa of the airway (188), leading to reductions in R_T (119) and the production of an isotonic transudate (8). In addition, *in vitro* studies of cultured bronchial surface epithelium have shown that, when inflamed, these cells switch from a mostly Na^+-absorptive to a mostly Cl^--secretory phenotype (49,74). Both of these studies further suggest that the increase in Cl^- secretion seen under inflammatory conditions is due, at least in part, to the activation or induction of apical Ca^{2+} dependent Cl^- channels (CLCA). Exciting new data from Nakanishi *et al.* (150) indicate that mouse CLCA-3 expression in the epithelium is important for the development of airway hyperresponsiveness, a feature characteristic of asthma. These investigators have shown that epithelial mCLCA-3 expression is upregulated in mice with airway hyperresponsiveness and that when suppressesed, using *in vivo* antisense adenoviral gene transfer, airway hyperresponsiveness and mucus overproduction are prevented. Furthermore, the authors demonstrate that transfection of human mucous epithelial cells with either mCLCA-3 or its closest human analog hCLCA-1 results in upregulation of mucin gene expression (MUC5AC). The importance of these studies was highlighted by the finding that hCLCA-1 expression is upregulated in human subjects with asthma, when compared to healthy controls (94). The relationship of CLCA expression to mucus secretion is unclear, but it had been reported, long before these studies, that in rabbit distal colon Cl^- secretion is associated with mucin secretion (88). Thus together, these studies provide strong evidence that altered anion transport is associated with inflammatory airway diseases

such as asthma, but further experimentation aimed at explaining how this leads to clinical illness is required.

2. Airway epithelial ion channels:

2.1 Anion channels:

All Cl^- channels transport other anions to some extent, in particular the physiologically relevant HCO_3^- ion (see section 1.3 and 1.4). Thus, referring to them by the more appropriate name "anion channels" is becoming more popular, but both names are common and often used interchangeably. To date four human protein families have been shown to contain anion channel members. Interestingly, three of these are clearly anion channel families (ClC, CLCA, GABA/Glycine receptors), while one family, the ABC transporters, typically do not conduct anions except for one member, CFTR. Members of each family, except the GABA/Glycine receptors, are expressed in airway epithelia. Furthermore, the great disparity between the numbers of biophysically identified versus molecularly identified anion channels suggests that there may be whole families of channel proteins that remain undiscovered (107). Thus, at least two additional anion channel activities exist in airway epithelia for which no molecular identity has been established, the Outwardly Rectifying Chloride Channel (ORCC) and the swelling-activated Cl^- channel ($I_{Cl,swell}$).

The CFTR channel. The fact that CFTR is a member of the ATP-binding cassette (ABC) transporter family is surprising. ABC transporters (for review see 117) act as ATP-dependent pumps and the inclusion of an anion channel into this family is

unexpected. As the name of the family suggests, all the members contain ABC domains with conserved Walker A and Walker B motifs. In addition a specific 16 amino acid sequence, termed the "ABC signature", is located between the Walker A and B loci. The sequence of the ABC signature can be found in the PROSITE database (http://www.expasy.ch/prosite/). The finding that CFTR expresses all three characteristics of ABC transporters confirms its membership in this family. Furthermore, the structure of CFTR, depicted in Figure 1.2, is very typical of ABC transporters (for review see 189). Two Transmembrane Domains, designated TMD1 and TMD2, are each composed of six α-helical transmembrane segments (TMS), which are collectively responsible for formation of the channel pore. The cytoplasmic C-terminus of each TMD contains a nucleotide binding domain (NBD), which is actually the ABC region from which the family derives its name, with NBD1 following TMD1 and NBD2 following TMD2. In most ABC transporters the purpose of these two domains is to hydrolyze ATP in order to fuel active transport, but in CFTR ATP hydrolysis allows for channel gating. A unique feature of CFTR is the cytoplasmic regulatory or R domain, which joins the C-terminal tail of NBD1 to the N-terminal head of TMD2. The R domain contains numerous consensus sequences for Protein Kinase A (PKA) phosphorylation, though these sites exist outside the R domain as well. This domain also contains several consensus sequences for Protein Kinase C (PKC), which appears to potentiate responses to PKA and may be essential for CFTR function (109,204). The cytoplasmic C-terminal tail of CFTR contains a five amino acid sequence known as a PDZ-binding domain. These domains can bind to PDZ domains found in several cytoplasmic proteins, the first of which discovered to bind to CFTR was the 50 kDa ezrin binding phosphoprotein (EBP50)

(190). As the name of this protein suggests, EBP50 can bind to ezrin, which in turn may bind PKA, thereby anchoring it to its target CFTR; ezrin can also bind to actin, thereby anchoring CFTR to the cytoskeleton (122). Thus, the PDZ binding domain of CFTR is thought to give it the ability to interact with other proteins.

Numerous experiments have clearly established that CFTR is a cAMP-dependent anion channel with a 6-10 pS Cl^- conductance and a linear current-voltage relationship in symmetrical Cl^- solutions (3,4,13,91,100). The halide permeability sequence of the CFTR channel was initially characterized as being $Br^- \geq Cl^- > I^- > F^-$ (3). However, it was later suggested that I^- may be more permeable than Cl^-, giving the sequence $I^- > Br^- > Cl^- > F^-$, but because I^- also blocks the pore under some conditions it gives the apparent halide permeability sequence initially described (205). Another important relationship is the ratio of Cl^-:HCO_3^- permeability through CFTR, which has been described as being 4-10 (79,130). This finding has posed a significant problem as it has been shown that some epithelia, which express CFTR channels as the dominant (or only) apical anion conductance, preferentially secrete HCO_3^- (see section 1.5). Using numerous polyatomic anions, with known dimensions, a number of studies have shown that the maximum diameter of anions permeable through CFTR is ~5.3 Å. This is the likely diameter of the pore at its narrowest region (98,131).

The array of pharmacological agents available for anion channel inhibition is relatively poor when compared to the tools available for cation channel investigation. Most of the drugs used to study anion channels are non-specific and/or of low potency (for review see 107). This fact has resulted in anion channel research that has lagged significantly behind research on cation channels. The most commonly used inhibitors of

CFTR channels are the arylaminobenzoates, including diphenylamine-2-carboxylate (DPC) and 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and the sulphonylureas, including glibenclamide and tolbutamide. However, DPC needs to be used in millimolar quantities and will block other anion channels, in particular several members of the ClC family and the $I_{Cl,swell}$, while NPPB will also inhibit CLCA channels even in micromolar quantities. The sulphonylureas do not fare much better, since they are used primarily as ATP-dependent K^+ channel (K_{ATP}) blockers in the treatment of diabetes mellitus. It is believed that these drugs can inhibit both CFTR and K_{ATP} channels because of their related structure; K_{ATP} channels are composed of Kir6 (see section 2.3) and Sulphonylurea Receptor (SUR) subunits, the latter being members of the ABC transporter family. Perhaps the most specific and potent CFTR blocker is suramin, but unfortunately it only works when applied to the intracellular aspect of CFTR (9). Similarly, disulfonic stilbenes, including 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), will block CFTR but only when applied from the intracellular aspect of CFTR. The latter drug in particular has been exploited as a means of distinguishing CFTR from many non-CFTR anion channels, which are sensitive to DIDS at their extracellular surfaces. However, a caveat to DIDS use is that this drug will also inhibit some transporters including anion exchangers and NBCs. Thus, the study of CFTR and the other anion channels has had to rely on the simultaneous use of numerous methods of distinguishing individual channels, including their pharmacological profiles, halide permeability sequences, regulatory factors, conductances, and current-voltage relationships.

While CFTR is clearly an anion channel, it was recognized even before its cloning that individuals with CF have multiple secondary defects that are not easily explained by

the lack of an anion conductance. Some of these secondary defects include dysregulation of ORCC activity (62,73) and hyperabsorption of Na^+ (21), which are discussed below. Thus, CFTR is not only an anion channel but also a regulatory protein, a fact reflected in its name – cystic fibrosis transmembrane conductance regulator.

The pattern of CFTR expression may be an important clue to understanding the role of this channel in the airway. While CFTR appears to be expressed down the entire length of the respiratory tract, beginning in the nose and ending in the alveoli, it is clear that this expression is not uniform. In the proximal airway the greatest expression of CFTR is in the serous cells of the submucosal glands (63), while the cells of the surface epithelium have been shown to contain only one or two mRNA transcripts each (212). This pattern of expression reflects the primarily secretory role of submucosal glands (218) while surface epithelium appears to be primarily absorptive (49,75). Less is known about the role of CFTR in the distal lung, but it has been shown that Clara cells express the majority of CFTR in the proximal bronchioles (64,121). In addition, functional studies have suggested that Clara cells are the primary mediators of CFTR-dependent water and electrolyte secretion in the distal airway (33,121). In the alveoli, CFTR appears to be important prenatally in the secretion of lung fluid (41,127,143). However, its role in adult alveoli, which are primarily absorptive, is not understood. One study suggests that CFTR expression in adult type II pneumocytes is insignificant (234), while another indicates that it plays a role in NaCl absorption (110). Recent studies on mouse lung *in vivo* and human lung *ex vivo* have shown that CFTR expression in the distal lung is important for the resolution of pulmonary edema, and that mutation or inhibition of CFTR impedes this process (65). Thus, the expression and role of CFTR varies along the airway epithelium.

The ORCC. The Outwardly Rectifying Chloride Channel (ORCC) has been biophysically described in the plasma membranes of many airway epithelial cell systems (62,73,187,226,227,233) and, before the cloning of CFTR, was even suspected of being the channel that is affected in individuals with CF (87). The channel is activated by depolarizing potentials, is of large conductance (~80 pS at depolarizing potentials, in excised patches), is regulated by cAMP, is inhibited by extracellular DIDS and is not activated by cell swelling. One of the most interesting properties of this channel is that it appears to be regulated by CFTR, such that when CFTR is absent the activation of ORCC by cAMP-dependent mechanisms is prevented (62,73,87,187). However, the mechanism, termed "conductance regulation", by which CFTR influences the ORCC is not fully understood. Patch clamp studies comparing nasal epithelia of CFTR knockout mice to wildtype demonstrated that the absence of CFTR alters neither the expression of ORCC, nor its current-voltage relationship, only its open probability (73). Further study in human bronchial cultures revealed that the activation of ORCC is achieved through CFTR-dependent ATP release and P_2-purinergic receptor (see section 3) stimulation (186). These authors' data also suggested that the negatively charged ATP is transported in an electrogenic manner through a channel that is, or is closely associated with, CFTR. These findings have led to vigorous debate over how ATP is secreted (see section 3.2), and thus, how ORCC is regulated.

The $I_{Cl,swell}$ Channel. The purpose of swelling activated anion channels ($I_{Cl,swell}$) is to mediate regulatory volume decrease (RVD). When a cell is exposed to a hypotonic environment $I_{Cl,swell}$ is activated, and often accompanied by the activation of swelling activated K^+ channels, resulting in Cl^- (and K^+) extrusion, which reduces intracellular

osmolarity, and therefore opposes cell swelling. In addition, other negatively charged osmolytes, such as taurine, glutamate or aspartate may also be extruded through what appears to be the same channels, in order to assist in achieving isotonicity (107). This prompted Strange *et al.* (101) to name the channels "volume-stimulated organic osmolyte/anion channels" (VSOAC). These channels are expressed in virtually every vertebrate cell. The channel is outwardly rectifying, of ~70 pS (at depolarizing potentials) conductance, extracellularly DIDS-sensitive, and often inactivated at depolarizing potentials (16,107). Channel gating is unique; when inactive the channel's open probability is zero, but when activated it opens suddenly, then displays almost no gating and thus, has an open probability near 1 (102). Channel activity is determined by $[Cl^-]_i$ (28,55), but whether this is sensed directly by the channel, or by some other process which then transduces the signal to the channel is unknown.

ClC channels. The first member of the voltage gated Cl^- channel (ClC) family was described in the electric organ of the torpedo ray (222). Subsequent characterization revealed that the channel behaves as if it contains two independently gated pores (147). Cloning of the channel (108), termed ClC-0, confirmed that it is a dimer, with each monomer contributing one independently gated pore (134). The number of transmembrane domains present was a matter of debate, until the three dimensional structure was elucidated by Dutzler *et al.* (60). They showed that there are 17 membrane associated α-helices that are often severely tilted with respect to the axis that runs perpendicular to the membrane. Thus, the widely varying lengths of the transmembrane domains and the fact that some do not fully span the membrane, explain why it was so difficult to determine their exact number through molecular and biophysical means.

To date the only high resolution three-dimensional structure of an anion channel is of the bacterial ClC (60,61). Thus, its structure provides the only direct insight into general anion channel features such as anion selectivity, and specific features characteristic of the ClC family, such as their two-pore structure and fast voltage-dependent gating. The selectivity filter is formed by the anti-parallel arrangement of α-helical dipoles, main-chain amide nitrogen atoms and by side-chain hydroxyl groups that all supply partial positive charges that are strong enough to bind permeant anions, but not so strong that they prevent conductance. The two pores of each ClC are not formed at the interface of subunits, as is the case for K^+ channels (56), but instead reside within each separate subunit. Voltage-dependent gating by ClC channels has been described previously (32,90,164,178) and is known to be highly dependent on extracellular [anion] and pH. These findings suggested the existence of a voltage-independent anion binding site in the pore, on the extracellular side of the selectivity filter, that becomes apparently voltage- and [anion]-dependent because the movement of the anion itself is dependent on these factors. The elucidation of the three dimensional structure of the ClC led to the description of a "swinging" glutamate residue that can replace an anion in this binding site, by virtue of its negatively charged carboxylate group, thereby blocking the channel (60,61). It was postulated that increasing the extracellular [anion] or depolarizing the membrane promotes competitive dislodging of the glutamate residue from the binding site, by the anion, and thus opens the channel. This model also explains the pH dependence of gating, since H^+ ions can protonate the carboxylate anion gate, dislodging it from the binding site and thereby activating the channel. Furthermore, this highly

localized "swinging gate" model explains how the fast-voltage dependent gates of each pore are able to function independently.

Nine ClC channels have been cloned in humans and fall into three groups based on their sequence homologies. The first set of channels comprised of ClC-1, ClC-2, ClC-Ka and ClC-Kb are plasma membrane anion channels, while the other two groups, encompassing ClC-3 to ClC-7, are normally found in intracellular membranes (107). Of the four plasma membrane ClC channels, only ClC-2 has a broad tissue distribution, and has been shown to be present in the apical membranes of airway epithelia (14,45,148). The channel is DIDS-insensitive, DPC-sensitive, and cAMP-dependent, and therefore may be experimentally mistaken for CFTR (45,185). The channel can be activated by arachidonic acid, extracellular acidification, amidation, and omeprazole in airway epithelial cells (45,46,211). Thus, ClC-2 channels may constitute a potential therapeutic target in CF if activation of these channels can be shown to significantly increase anion secretion in mature tissues.

The recent cloning and characterization of a novel ClC-3 splice variant, designated ClC-3B, has generated much interest since the channel appears to display many of the biophysical features attributed to the ORCC (153). The finding that the ClC-3B channel displays outward rectification, is activated by depolarizing potentials, is of large conductance, is expressed primarily in epithelial cells, is inhibited by extracellular disulfonic stilbenes, and is not activated by cell-swelling, suggests that this channel may be the ORCC (153). In addition, the ClC-3B (153) and native ORCC (62) current-voltage relationships appear to be almost identical. Interestingly, ClC-3B contains a PDZ binding domain that interacts with EBP50 (153). Furthermore, cotransfection of ClC-3B, EBP50

and CFTR into C127 cells results in the expression of ORCC-like channels that can be activated by cAMP-dependent mechanisms, but are cAMP insensitive if CFTR is omitted or replaced with ΔF508 CFTR. Thus, these findings suggest that like native ORCC, ClC-3B is regulated by CFTR, and also proposes a mechanism by which this regulation may occur. However, subsequent studies have revealed that consistent with the generally accepted patterns of ClC expression, this channel appears to be expressed only in intracellular membranes of airway epithelia, particularly in the Golgi where it interacts with a small pool of CFTR (76). Thus, this channel is not likely to be the native ORCC. However, the striking similarities between the two channels suggest that the ORCC may be a closely related protein.

CLCA channels. Native Ca^{2+}-dependent Cl^- channels are present in the apical membranes of airway epithelial cells (5). These channels, and therefore anion secretion, can be activated by factors that elevate intracellular Ca^{2+}, such as ionomycin, bradykinin or ATP, and their expression is conserved in CF airway epithelia (5,83). It is still unclear if the Ca^{2+} sensitivity of these channels is direct, or if it is dependent on an intermediary, such as PKC or calmodulin kinase II (CaMK II). Single-channel studies revealed a wide range of conductances from 1-70 pS (107), likely indicating great molecular diversity. Whole-cell patch clamp studies suggest that the current voltage-relationship is linear or slightly outwardly-rectifying (5), and that these channels are sensitive to inhibition by extracellular DIDS, NPPB and niflumic acid. The importance of these channels was proposed in studies by Grubb *et al.* (83) who found that in the CF mouse, nasal epithelial Ca^{2+} dependent Cl^- secretion is upregulated. This suggests that the presence of an alternative Ca^{2+} dependent Cl^- channel may be the reason that mice are resistant to airway

disease in the absence of CFTR. This was supported by the finding that the occurrence of this Ca^{2+}-dependent Cl^- channel activity in a CF mouse organ was inversely correlated with the severity of the disease in that organ (37).

Four human members of the Ca^{2+} dependent Cl^- channel (CLCA) protein family have been cloned to date. Of these, the only one found to be expressed at the plasma membrane of airway epithelia is hCLCA2 (also found in mammary tissue) (84). Studies by Gruber *et al.* (70) are the only ones describing the channel. It was found to contain seven consensus sequences for phosphorylation by the Ca^{2+}-dependent enzyme PKC, but none for CaMK II. Furthermore, it was found to contain five transmembrane domains, but it is unknown if this is a characteristic of this family as a whole, since this is the only CLCA channel whose topology has been systematically investigated. Functional expression studies revealed a slightly outwardly rectifying whole-cell anion conductance, which could be activated by ionomycin and inhibited by extracellular DIDS or niflumic acid. Though expression of this protein results in activity characteristic of native Ca^{2+}-dependent Cl^- channels, it is possible this, or any other CLCA protein cloned, simply activates the as yet unidentified native channel. Mutagenesis studies in particular will be useful in this determination, if it can be shown that changes in the sequence of this protein results in changes in the biophysical properties of the channel such as its halide permeability sequence.

2.2 The epithelial Na^+ channel:

By the time the epithelial Na^+ channel (ENaC) was cloned, first in rat (26,27), then in humans (144,145), its role in ion transport, regulation by mineralocorticoids and its

sensitivity to inhibition by amiloride was well characterized. Upon cloning, the channel was found to belong to an emerging family of proteins termed degenerins, which are now known collectively as the ENaC-Degenerin family. Interestingly, while ENaC seems to be expressed exclusively in epithelia, the degenerins are found mostly in neurons (2). The fact that this Na^+ channel is distinct from the family of voltage-gated Na^+ channels in the nervous system reflects its distinct physiological role. While the latter are involved in the generation of transient electrical signals in excitable tissues, the former is involved in sustained vectorial ion transport across epithelia.

Cloning led to the discovery that the channel is composed of three subunits, designated α, β and γ. While the α-subunit can form a functioning channel on its own, its association with β and/or γ subunits results in the generation of functional diversity (72,146). Though the β and γ subunits have been traditionally regarded as incapable of forming functional channels alone, recent evidence indicates that the lack of the α-subunit simply results in a reduced ability of the channel to assemble and traffic to the plasma membrane (17). The stoichiometry of the subunits has yet to be agreed upon. At least two different hypotheses have been proposed with α:β:γ ratios of 2:1:1 (68,120) and 3:3:3 (196). Each subunit has two transmembrane (TM) domains, with short cytoplasmic N and C- terminal tails, and a long extracellular loop that makes up half of the protein, a characteristic feature of ENaC-Deg family members (114,171). While members of the voltage-gated Na^+ channel family have selectivity filters composed of extracellular loops, the ENaC selectivity filter is composed of amino acids contributed by the transmembrane domains, particularly TM2 (113,171,182,219).

The mechanisms of ENaC regulation have garnered much attention in light of findings that CF tissues display abnormal Na^+ absorption. Most studies in transfection systems (199), airway epithelia (137) and intestinal epithelia (138) indicate that CFTR inhibits ENaC activity. The mechanism of this regulation was suggested in studies by Stutts *et al.* (200), in which fibroblasts were transfected with ENaC ± CFTR. They found that cAMP-dependent activation of ENaC occurs in the absence of CFTR, and cAMP-dependent inhibition in its presence. In contrast, studies by Reddy *et al.* (168) have shown that in human sweat duct, ENaC can be activated by cAMP but only if CFTR is also activated. Several mechanisms by which CFTR and ENaC interact have been suggested: direct physical interaction via PDZ binding domains, autocrine signaling via ATP (or another unidentified molecule), CFTR-regulated exo/endocytosis, via cytoskeletal proteins, and through $[Cl^-]_i$ (123,184). Other studies have shown that ENaC may be regulated by proteases/antiproteases (23,54); Protein Kinase C (PKC) inhibition (123); $[Na]_i$ feedback inhibition through a mechanism dependent on G_o-protein and Nedd4 ubiquitin ligase (53,118); and $[Cl]_i$ feedback inhibition through a mechanism dependent on G_i-protein and independent of Nedd4 ubiquitin ligase (53,118). The regulation of ENaC by CFTR is particularly significant in relation to the isotonic ASL model of ion transport. This model suggests that lack of CFTR leads to overactive ENaC-dependent Na^+ absorption, ASL dehydration, ineffective mucociliary clearance and infection typical of CF (see section 1.5).

One of the most important physiological roles of ENaC is to mediate the absorption of fluid from the alveoli at birth, and to keep them nominally fluid free from then on, in order for efficient gas-exchange to occur. The primary role of the fetal alveolar

epithelium is to secrete fluid in a CFTR dependent manner (152). Fetal rat alveolar pneumocytes express low levels of a non-selective cation channel (NSC) with a conductance of 23-27 pS, and only begin to express high levels of typical ENaC activity (6 pS, P_{Na}/P_K = 80) shortly before birth (104,156,157). Interestingly, experiments performed on adult rat type II pneumocytes grown submerged under conditions of reduced oxygen delivery or in the complete absence of corticosteroids were found to express the same NSC channel, and lack typical ENaC activity (104). In contrast, cells grown on permeable filters at an air-liquid interface and with high concentrations of dexamethasone expressed typical ENaC activity (104). Similar results were observed in the human type II alveolar pneumocyte A549 cell line (126). This was explained when it was found that the NSC channel was actually the α-subunit of ENaC expressed in the absence of the β and γ subunits, while channels that displayed typical ENaC activity were composed of α, β and γ subunits (104). Thus, it appears that increased oxygen delivery and glucocorticoids are responsible for upregulation of typical ENaC channels late in gestation. At birth, an increase in circulating catecholamines activates Na^+-absorption and results in absorption of the lung fluid. A lack of ENaC activity at birth, either through pharmacological inhibition, genetic knockout, or a failure to upregulate as occurs in premature infants, results in neonatal respiratory distress syndrome (151). These experiments suggest that one of the mechanisms by which prenatal glucocorticoid therapy reduces the severity of respiratory distress in premature infants is through upregulation of ENaC channels.

2.3 K^+ channels:

K^+ channels are the least understood channels in airway epithelia. While ENaC and Cl^- channels, particularly CFTR, garner all the research attention, K^+ channels are largely overlooked. However, their importance cannot be understated; without K^+ channel activity sustained anion secretion cannot occur, as this would depolarize the cell and eliminate the electrochemical driving force (136). Furthermore, there is evidence that K^+ secretion can be stimulated in some human airway epithelial preparations (36).

There are three known human K^+ channel families that can be differentiated based on their molecular structures. The voltage dependent K^+ channels (K_v) are tetramers of subunits each composed of 6 transmembrane segments (TMS) and one pore (P) domain, a loop of amino acids with a conserved sequence that forms the selectivity filter. The inwardly rectifying K^+ channels (K_{ir}) are also tetramers, with each subunit composed of 2 TMS and 1 P domain. In contrast the "leak" K^+ channels are dimers, with each subunit composed of 4 TMS and 2 P domains. To date, two types of K^+ channels have been described in airway epithelia – cAMP- and Ca^{2+}-dependent K^+ channels, both of which belong to the K_v channel family.

If basolateral K^+ channels need to be activated at the same time as apical anion channels in order for sustained anion secretion to occur, it would make sense that the same regulatory pathways would activate them. Thus, since the cAMP-dependent CFTR is the predominant apical anion channel, it was gratifying to find that a portion of basolateral K^+ channels are also cAMP-dependent (43,135,140,220). In 1988 Takumi *et al.* (206) cloned a small protein, known today as KCNE1, with only one putative transmembrane domain. Surprisingly, when expressed in heterologous systems, a K^+

channel was formed whose activity was very similar to that of a channel mutated in individuals with a heritable cardiac arrhythmia known as long QT syndrome (69,206). This was explained when it was found that KCNE1 activates endogenous K^+ channels (7), and was followed by the finding that the human homolog of this endogenous channel is KCNQ1, also known as K_vLQT1 (181). Thus, mutations in KCNQ1, or its regulatory subunit KCNE1, result in long QT syndrome (181). When these channels were expressed in *Xenopus* oocytes, a cAMP-dependent K^+ channel could be detected that was sensitive to inhibition by a family of compounds, the prototype of which is chromanol 293B (15,201). At the same time, other studies had shown that endogenous epithelial cAMP-dependent K^+ channels are sensitive to inhibition by the same compounds (51,133). Eventually, Mall *et al.* (140) showed that KCNQ1 is responsible for the basolateral cAMP-dependent K^+ channel in human airway epithelium. However, when KCNQ1/KCNE1 are expressed, strongly outwardly rectifying currents are produced, and it was unclear how these channels could participate in anion secretion when epithelia never experience such strong depolarization. This was resolved when Schroeder *et al.* (183) discovered a novel KCNE3 protein that associated with KCNQ1 instead of KCNE1 in epithelium to form a cAMP-dependent K^+ channel active at physiological epithelial membrane potentials that was sensitive to inhibition by chromanol 293B. A number of studies have now implicated KCNQ1\KCNE3 as the channel likely responsible for the basolateral cAMP-dependent K^+ conductance in airway epithelia (43,78,135).

Since Ca^{2+} can activate anion secretion, it was not surprising to find that basolateral Ca^{2+}-dependent K^+ channels are also found in airway epithelia. The Ca^{2+}-dependent family of K^+ channels was initially divided into two groups, small conductance (SK) and

big conductance (BK) channels. However, while SK channels appear to be expressed only in the nervous system, BK channels have a wider distribution (92). In particular, BK channel activity has been demonstrated in type II alveolar pneumocytes (172) and bronchial epithelium (208) where its activity can be selectively inhibited using iberiotoxin. The recent cloning of the fourth member of the SK family revealed that it was expressed predominantly in non-excitable tissue, and had a relatively low homology to the other members of this family (112). Thus, it was proposed that this channel forms its own subfamily of intermediate conductance Ca^{2+}-dependent K^+ (IK) channels, and is now known as IK-1. It has been shown that airway epithelia express this clotrimazole sensitive channel at their basolateral membranes, and that it is particularly important for Ca^{2+}-dependent anion secretion (43,139,228).

Recently, a novel role has been proposed for basolateral K^+ channels in the airway. Studies showed that cAMP stimulates HCO_3^- secretion in Calu-3 cells, a model of submucosal gland epithelium, but subsequent application of basolateral 1-ethyl-2-benzimidazolone (EBIO) switches the epithelium to Cl^- secretion (52,207). The authors proposed that basolateral EBIO, a K^+ channel activator (48), hyperpolarizes the cell, which promotes Cl^- secretion but inhibits the basolateral NBC and therefore HCO_3^- secretion. NBC function is inhibited by hyperpolarization because it is electrogenic, translocating a net negative charge into the cell. Thus, K^+ channels also appear to be involved in the regulation of the type of anion secreted by the airway epithelium.

It is still unclear if activation of basolateral K^+ channels may have a therapeutic role in CF, for a number of reasons. First, there is no benefit to modulating basolateral processes if there is a complete lack of an apical anion conductance. Second, opening of

basolateral K^+ channels in CF may be counterproductive since cell hyperpolarization stimulates Na^+ absorption, which is already overactive. Lastly, while the activation of basolateral K^+ channels activates Cl^- secretion, it appears to inhibit HCO_3^- secretion and thus may exacerbate the illness.

3. Adenosine and the airway epithelium:

3.1 Adenosine signaling:

The purine nucleoside adenosine is a ubiquitous paracrine/autocrine mediator that is known to regulate airway epithelial ion transport, and therefore the quantity and composition of ASL (31,35,40,95,96,149,163,177). Adenosine mediates its effects via stimulation of P_1-purinergic receptors of which there are four known subtypes, designated A_1, A_{2A}, A_{2B}, and A_3. These belong to the seven transmembrane domain superfamily of G-protein coupled receptors (GPCR), which has over one thousand members (please see GPCR database at http://www.gpcr.org/7tm/).

Members of the GPCR superfamily transduce their signals across the plasma membrane through G-proteins, consisting of α, β and γ subunits. The α-subunit is the most important determinant of the function of a given G-protein. Thus, G-proteins have been grouped into four main subfamilies based on the sequence homologies of their α-subunits: G_s, adenylyl cyclase activators; $G_{i/o}$, adenylyl cyclase inhibitors; G_q, phospholipase C activators; and G_{12}, which stimulate Rho-dependent signaling pathways (71). Regulation of ion channel function by G-proteins is most frequently attributed to $G_{s/i/o}$ effects on adenylyl cyclase, as well as through G_q effects on protein kinase C (PKC)

and changes in intracellular Ca^{2+} (154). In addition, G-proteins have been shown to regulate some ion channels directly (42,59,96). Adenosine receptors associate with the following G-proteins: A_1 with $G_{i/o}$; A_{2A} with G_s; A_{2B} with G_s, G_q; and A_3 with G_i, G_q (167).

The expression of A_{2A} and A_{2B} receptors in airway epithelia is well established (12,31,35,40,125), but the importance of A_1 receptors is controversial (111,125,142), suggesting disparate expression in various cell types. A_3 receptors have been shown to be expressed in total human lung, but it is unknown if they play any role in the epithelium (179). Given that the most widely expressed adenosine receptors in airway epithelia are G_s coupled, it is not surprising that stimulation typically leads to cAMP- and CFTR-dependent anion secretion (35,40,125). Other investigators have shown that arachidonic acid or changes in intracellular Ca^{2+} may also be involved in adenosine responses in some cell types (31,40).

There has been little investigation into identifying the targets of adenosine signaling. Since adenosine functions primarily through cAMP-dependent pathways, most investigators assume it will have similar effects to the well characterized responses to β-adrenergic agonists or cAMP elevating agents such as forskolin. This approach is supported by numerous studies that show that adenosine activates CFTR (40,95,96). Significantly, adenosine has also been shown to stimulate anion secretion in tissues expressing clinically relevant CFTR mutants such as R117H (35). Thus, therapeutic strategies aimed at activation of adenosine receptors may be of benefit to individuals with CF.

Interestingly, while adenosine activates CFTR as efficiently as forskolin, it results in the generation of much less cAMP (35,95). This suggests that adenosine signaling to CFTR is compartmentalized. This was demonstrated functionally in studies by Huang *et al.* (95), who showed that 1 μM adenosine (~ED_{50}) stimulates CFTR channels when applied to the pipette solution of a cell-attached patch, while even 100 μM adenosine (maximal dose) applied to the bath solution had no effect on channels within the patch. Thus, CFTR channels can only be activated by adenosine receptors also in the patch. This appears to occur as a result of the formation of multi-protein complexes that contain all of the factors necessary for signal transduction and their targets. A-kinase anchoring proteins (AKAPs), such as ezrin, which anchors PKA to its physiological targets, are an example of the "glue" proteins that hold these complexes together. In whole-cell patch clamp studies, intracellular perfusion of Ht-31, a peptide that binds and inhibits AKAPs, prevents adenosine from activating CFTR (96). Thus, it appears that macromolecular complexes are important for the compartmentalization of signal transduction factors that allow adenosine to activate CFTR.

3.2 Adenosine homeostasis:

Because adenosine mediates many physiological functions, any therapy that seeks to exploit adenosine signaling directly has the potential for a broad spectrum of side effects that would likely preclude its use. This has led to the search for tissue specific ways of influencing adenosine signaling. In particular, pathways of adenosine transport and metabolism may represent potential therapeutic targets. In general, adenosine is rapidly metabolized intracellularly, so that its concentration is kept very low. Adenosine

nucleotides are secreted by cells, which are metabolized to adenosine extracellularly, and are then available for interaction with cell surface receptors. Signaling is terminated when adenosine is taken up by cells and metabolized.

The pathways of intracellular adenosine metabolism are well known. Adenosine is normally metabolized by adenosine kinase to AMP, thereby directing it to cellular respiration pathways that will eventually produce ATP. Alternatively, intracellular adenosine can be metabolized by adenosine deaminase to produce inosine, which is essentially a waste product that is eventually converted to uric acid by the liver and excreted in the urine. These pathways operate very efficiently and ensure that intracellular adenosine concentrations are usually kept at almost negligible levels. Normally the adenosine kinase pathway of adenosine metabolism predominates because the Michaelis-Menten constant (K_m) for adenosine kinase is significantly lower then that for adenosine deaminase (50,209). However, if adenosine concentrations become too high, as can occur under hypoxic conditions when cellular respiration is inhibited, adenosine deaminase activity predominates over adenosine kinase activity. This is because the reaction catalyzed by adenosine deaminase has a higher maximal velocity (V_{max}) then that catalyzed by adenosine kinase (50,209). Thus, under normal conditions adenosine kinase directs adenosine towards cellular respiration by virtue of its lower K_m, while under conditions of excess adenosine production, adenosine deaminase diverts much of the adenosine towards excretion, by virtue of its higher V_{max}.

Adenosine is not normally secreted by cells, because its intracellular concentration is negligible. Instead all human cells have the ability to secrete ATP, which is then the source of extracellular adenosine. Secretion can be induced by mechanical stimulation

(85,149) or by hypotonicity (22). Once secreted, ATP is metabolized by phosphatases to adenosine, which is then available for interaction with P_1-receptors. In addition, P_2-purinergic receptors can be stimulated directly by ATP, but are insensitive to adenosine. The mechanism by which ATP is secreted is controversial. This controversy began when Schwiebert *et al.* (186) showed that the ORCC is activated by CFTR-dependent ATP secretion (see section 2.1). The authors' further suggested that the negatively charged ATP is transported in an electrogenic manner through a channel that may be CFTR itself. These findings were quickly countered in studies by Reddy *et al.* (169), which failed to detect any CFTR-dependent ATP conductance in native airway epithelium, sweat duct, heterologous expression systems, or planar lipid bilayers containing CFTR. This was followed by numerous studies, which either supported (159,170,202,210) or refuted (85,86,129,169) the existence of a CFTR associated ATP conductance. Thus, while the mechanism of ATP secretion remains unknown three possibilities, which may or may not be CFTR dependent, have been proposed: via a channel, via a transporter or via exocytosis.

Cells also have the ability to secrete cAMP, which like ATP can be converted to adenosine extracellularly. However, the mechanisms by which this occurs are even less well understood. It is clear however, that cAMP efflux occurs ubiquitously in prokaryotes and eukaryotes when adenylyl cyclase activity is stimulated (155). Furthermore, studies have suggested that ABC-transporters, particularly CFTR, may be involved in this phenomenon (155).

Extracellular nucleotides are broken down to AMP by the activity of nonspecific phosphatases. This AMP can then be converted to adenosine by the activity of membrane

bound ecto-5'-nucleotidase also known as CD73 (161,198). In addition, adenosine can come directly from surrounding non-epithelial cells. In the lung, mast cells are known to secrete significant amounts of adenosine (12). This contribution is particularly important under inflammatory conditions, such as those seen in asthma, when mast cell degranulation is occurring. This was highlighted in studies by Driver *et al.* (58) who showed that the adenosine concentration in the bronchoalveolar lavage fluid of asthmatics is elevated when compared to controls.

3.3 Nucleoside transporters:

Adenosine signaling is terminated by its transport into the cell, via ubiquitous nucleoside transport processes. Unlike ATP transport, the mechanisms by which adenosine is transported are well understood. Knowledge of these processes is being applied therapeutically in the treatment of various illnesses including cardiovascular disease. For example, inhibitors of nucleoside transport are being used to promote coronary arterial vasodilation by potentiating endogenous adenosine signaling (10). However, there has been surprisingly little characterization of these processes in airway epithelium, despite their therapeutic potential.

In human cells two types of nucleoside transport processes have been described, one equilibrative (Na^+-independent) and one concentrative (Na^+-dependent). The equilibrative transporters are facilitative carriers that translocate adenosine, and other nucleosides, down their normally inwardly directed concentration gradients. These can be further subdivided into equilibrative-sensitive (*es*) and equilibrative-insensitive (*ei*) activities, based on sensitivity to inhibition by nanomolar concentrations of the drug

nitrobenzylmercaptopurine ribonucleoside (NBMPR), also known as nitrobenzylthioinosine (NBTI). Both *es* and *ei* can be inhibited by the coronary vasodilators dipyridamole, dilazep and draflazine, some of which are being used clinically. The *es* transport process appears to be expressed ubiquitously and both processes display broad permeant selectivity for purine and pyrimidine nucleosides (24). Two human members of the equilibrative family were cloned in 1997; human equilibrative nucleoside transporter 1 (hENT1) displays *es* activity (80), and human equilibrative nucleoside transporter 2 (hENT2) displays *ei* activity (81). Together these form a novel family of membrane proteins made up of 11 α-helical transmembrane domains, with intracellular N-terminal tails (203). The hENT1 and hENT2 transporters display distinct kinetic properties; in addition, hENT2 is able to transport nucleobases (230). Thus, they may play complementary but distinct roles. It is unclear if there are any other plasma membrane proteins that are members of this family. The recently cloned putative hENT3 has failed to display any nucleoside transport activity when expressed in *Xenopus* oocytes (97). This may be explained by specific motifs within the protein that suggest it may be expressed exclusively in intracellular membranes (180).

The concentrative nucleoside transporters are Na^+-dependent symporters that can transport nucleosides into the cell, even against concentration gradients. Five concentrative transport activities have been characterized in human cells. These can be subclassified into two categories based on their sensitivity to NBMPR: concentrative-sensitive (*cs*), and concentrative-insensitive (*ci*). These can then be further subclassified, based on their permeant selectivities into: *cit*, *cif*, *cib*, *cs*, and *csg* activities (for review see 30). To date three human proteins that display concentrative nucleoside transport

activity have been cloned and form a novel subfamily of the Na^+-cotransporter superfamily, the human concentrative nucleoside transporters (hCNT). When expressed in *Xenopus* oocytes these display: *cit* activity, with pyrimidine and adenosine selectivity, by hCNT1 (175); *cif* activity, with purine and uridine selectivity, by hCNT2 (176); and *cib* activity, with purine and pyrimidine selectivity, by hCNT3 (174). Interestingly, adenosine and uridine appear to universal permeants of these transporters. However, the very low V_{max} observed for rat CNT1 mediated transport of adenosine suggests that this nucleoside acts more like a physiological inhibitor of this transporter (66,229). A membrane topology of 13 α-helical transmembrane domains, and a cytoplasmic N-terminal tail characterize these proteins (89). The molecular identity of *cs* and *csg* activities has yet to be determined. Unlike the equilibrative transporters, hCNT expression appears to be limited to specialized tissues such as intestinal and renal epithelia, liver, choroid plexus, splenocytes, macrophages and leukemic cells (10,29,231).

OBJECTIVES AND HYPOTHESES

This thesis deals with two aspects of adenosine signaling in airway epithelia. First, the factors involved in adenosine signaling and their relative contributions are characterized. The application of exogenous adenosine is known to stimulate airway epithelial anion secretion, but it is unknown what determines its endogenous levels and, therefore, what its physiological role is. Furthermore, the use of drugs that target adenosine-dependent processes indirectly, by modulating its pathways of transport and metabolism, have been employed in the treatment of illnesses outside of the lung as a means of conferring specificity to this otherwise ubiquitous physiological mediator. Thus, similar characterization of the pathways of adenosine signaling and metabolism in the airway epithelium may lead to the identification of potential therapeutic targets for the treatment of pulmonary diseases such as Cystic Fibrosis (CF).

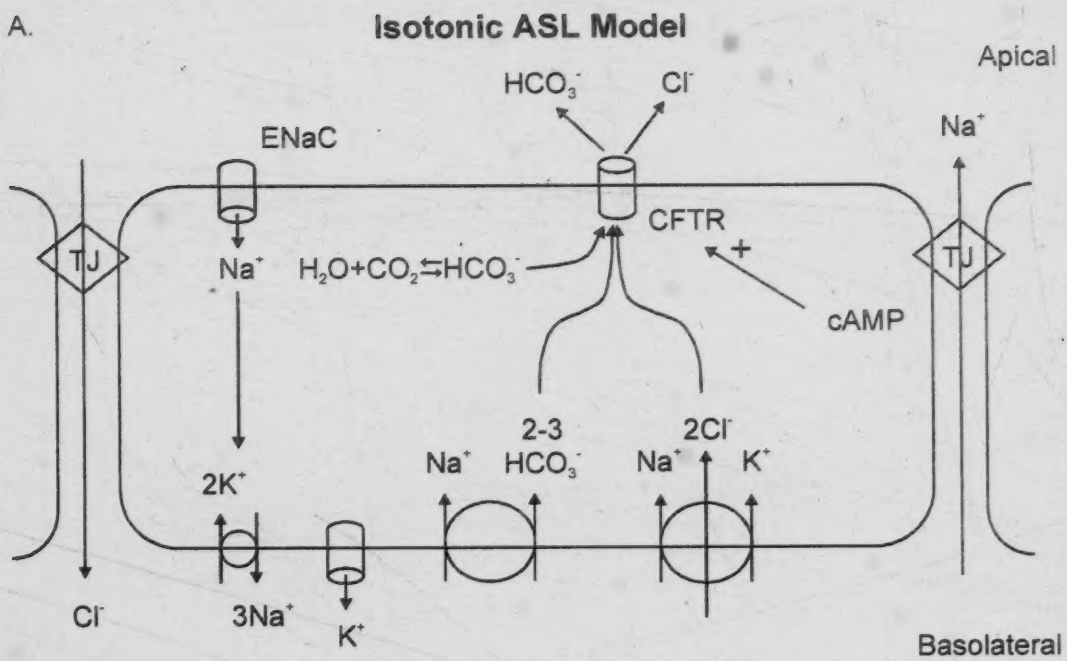
The identification of ion channel targets for adenosine signaling in airway epithelia constitutes the second aspect of this thesis. The hallmark of CF airway disease is a reduction in epithelial anion secretion, which may be remedied by the application of exogenous adenosine (35). Therefore, identification of the adenosine-dependent ion channels that compensate for the lack of CFTR and characterization of their role in ion transport may have therapeutic implications.

Therefore, the objectives of this study were:

1. To identify the receptors responsible for adenosine signaling in airway epithelia, their localization, and their relative contributions to anion secretion.
2. To identify the factors that are involved in determining endogenous extracellular adenosine concentrations.
3. To investigate the ion channels regulated by adenosine, their significance in anion secretion and their potential as therapeutic targets in the treatment of CF.

We hypothesized that:

1. Adenosine receptor expression is polarized and varies along the airway, in surface epithelium, submucosal glands and alveoli.
2. It is possible to modulate adenosine-dependent ion transport by modulating the pathways of adenosine transport and metabolism.
3. Adenosine activates ion channels that compensate for a reduction in CFTR activity.


A.
Isotonic ASL Model
Apical
HCO3-
Cl-
ENaC
Na+
CFTR
TJ
Na+
H2O+CO2⇆HCO3-
+
TJ
cAMP
2-3
HCO3-
2Cl-
Na+
K+
2K+
Cl-
3Na+
K+
Basolateral

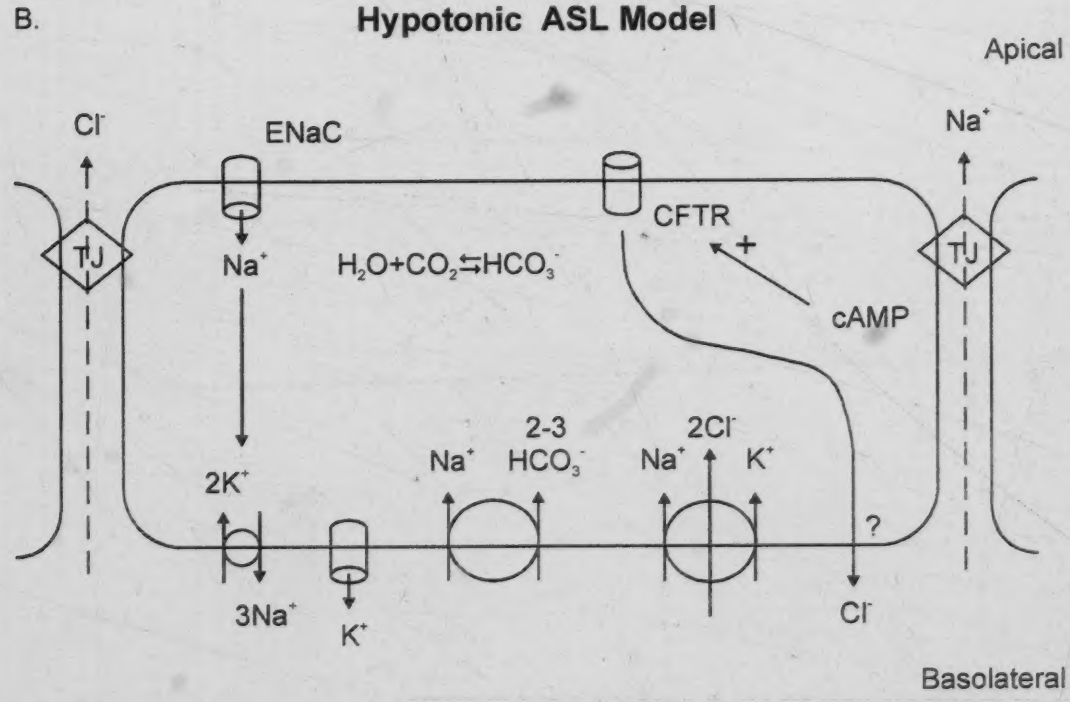

B.
Hypotonic ASL Model
Apical
Cl-
ENaC
Na+
CFTR
TJ
Na+
H2O+CO2⇆HCO3-
+
TJ
cAMP
2-3
HCO3-
2Cl-
Na+
K+
2K+
?
3Na+
K+
Cl-
Basolateral

Figure 1.1

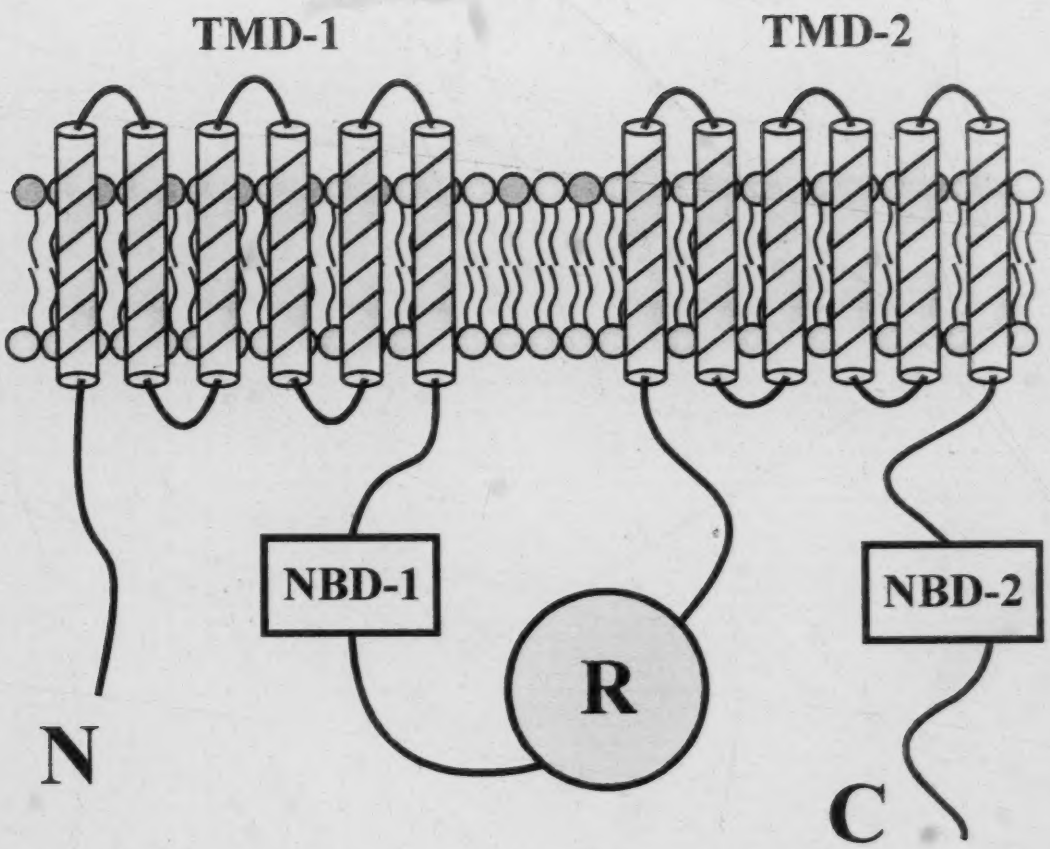


Figure 1.2

FIGURE LEGENDS

Figure 1.1

The isotonic and hypotonic ASL models of airway epithelial ion transport. The schematic depicts a cell representative of an epithelial monolayer with the apical membrane at the top, the basolateral membrane at the bottom and tight junctions (TJ) between adjacent cells. Full arrows indicate the major pathways of ion transport; dashed arrows indicate limited, or insignificant pathways. ***A***: The isotonic ASL model proposes that secretion occurs when Cl^- and HCO_3^- are transported into the cell via basolateral Na^+-dependent cotransporters, with an additional pathway of HCO_3^- production inside the cell, and exit across the apical membrane via CFTR channels that can be activated by cAMP. Na^+ will pass through the tight junctions in order to balance the charge separation created. At the same time transcellular absorption of Na^+ occurs via apical ENaC channels and the basolateral Na^+/K^+-ATPase. Cl^- will pass through the tight junctions in order to balance the charge separation created. Basolateral K^+ channels determine membrane potential and thus the magnitude of secretion/absorption, and the type of anion (Cl^- or HCO_3^-) secreted. Net salt secretion will result if transcellular anion secretion dominates over transcellular Na^+ absorption, resulting in an osmotic gradient favouring fluid secretion. Transepithelial water flux (not shown) can occur via paracellular or transcellular (Aquaporin-mediated) pathways. ***B***: The hypotonic ASL model proposes that ENaC and the Na^+/K^+-ATPase are responsible for Na^+-absorption, which creates an electrochemical gradient favoring transcellular Cl^- absorption through apical cAMP-dependent CFTR channels and an

unidentified basolateral anion channel (?). In this model, ion movements across the tight junctions are limited, thus the ASL becomes hypotonic.

Figure 1.2

Topology of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). CFTR is composed of twelve transmembrane segments divided into two transmembrane domains (TMD-1 and -2), which are presumed to make up the pore of the anion channel. The two nucleotide binding domains (NBD-1 and -2) are responsible for ATP hydrolysis, necessary for normal gating, and are characteristic of ABC transporter protein family members. In addition, a regulatory (R) domain is found between NBD-1 and TMD-2. This domain contains multiple consensus sequences for both PKA and PKC phosphorylation.

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CHAPTER 2

Regulation of K^+ current in human airway epithelial cells by exogenous and autocrine adenosine

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INTRODUCTION

The purine nucleoside, adenosine is an important mediator of many physiological functions, including transepithelial electrolyte secretion in human airways. Adenosine mediates its effects through the activation of high affinity receptors, A_1 and A_{2A}, that are physiologically relevant, and low affinity receptors, A_{2B} and A_3, that could play a crucial role under inflammatory conditions (34). Stimulation of adenosine receptors activates anion conduction via both CFTR-dependent and -independent pathways (11,26,39), although the underlying mechanisms are not fully understood.

The magnitude of the effect of adenosine on ion transport is related to its concentration in the vicinity of its cell-surface receptors. In healthy subjects, the average adenosine concentration in airway surface liquid is ~60 μM, while in patients with asthma it is ~200 μM (14). The increased adenosine concentration in asthmatic patients raises two important questions: what is the source of adenosine and how are its levels controlled? One factor that is believed to control adenosine concentrations is the balance between the activities of enzymes that catalyze its synthesis and those that catalyze its metabolism. Adenosine is produced by the action of membrane-bound 5'-nucleotidase on extracellular AMP, which is itself produced by the action of nonspecific (alkaline or acidic) phosphatases on ADP and ATP (35). Conversely, the metabolism of adenosine is mediated by the action of either adenosine kinase or adenosine deaminase, resulting in the conversion of adenosine to AMP or inosine, respectively (35). Since the majority of adenosine synthesis occurs extracellularly while most of its metabolism occurs

intracellularly, inwardly-directed transport of adenosine across the plasma membrane is also an important determinant of its extracellular concentration (8).

Because adenosine and other nucleosides are relatively hydrophilic, their uptake and release from cells depends upon specialized nucleoside transporter proteins present in the plasma membrane (8). These are members of the concentrative (Na^+-dependent) nucleoside transporter (CNT) and equilibrative (Na^+-independent) nucleoside transporter (ENT) families (1,7,46). Molecular cloning studies, in humans and rodents, have identified three distinct members of the concentrative family (CNT1, CNT2, and CNT3) and two members of the equilibrative family (ENT1 and ENT2). Human CNT1 and CNT2 both transport uridine and certain uridine analogs, but are otherwise selective for either pyrimidine (hCNT1) or purine (hCNT2) nucleosides, except for modest transport of adenosine by hCNT1 (37,38,41). In contrast, hCNT3 transports both purine and pyrimidine nucleosides (36). Human ENT1 and ENT2 also transport both purine and pyrimidine nucleosides and are distinguished functionally by a difference in sensitivity to inhibition by nitrobenzylthioinosine (NBTI), hENT2 being NBTI-insensitive (12,18,19). They also differ in sensitivity to inhibition by the coronary vasodilators dipyridamole, dilazep, and draflazine (hENT1 > hENT2), and by the ability of hENT2 to transport nucleobases as well as nucleosides.

The concentrative (inwardly-directed) nucleoside transporters, of rodents and humans, are expressed in specialized cells such as intestinal and renal epithelia, liver, choroid plexus, splenocytes, macrophages and leukemic cells (1,7,46). The equilibrative (bidirectional) nucleoside transporters have generally lower substrate affinities than concentrative transporters and occur in most, possibly all, human and rodent cell types.

While the role of nucleoside transport in the control of transepithelial anion secretion is unknown, it has been recently suggested that CNT2 could play an important role in the regulation of Na^+ reabsorption in cultured rat epididymal epithelium (28).

The aim of the present study was to characterize the role of adenosine receptors and transporters in the regulation of ion transport in the human airway epithelial cell line, A549. These cells exhibit metabolic and transport properties consistent with type II pneumocytes and, since they do not express CFTR (16), constitute a convenient model for studying CFTR-independent regulation of ion transport by adenosine. Our data show that regulation of adenosine receptor function by hENTs controls ion transport in A549 cells.

MATERIALS AND METHODS

A) Cell culture:

A549 cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 50 μg/ml gentamycin sulfate, 60 μg/ml penicillin-G and 100 μg/ml streptomycin. Cells were maintained in T25 tissue-culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 5% CO_2 in air. Confluent cell layers were passaged using saline solution containing 0.05% trypsin and 0.02% EDTA. For adenosine transport experiments, cells were seeded at a density of 10^6 cells/cm^2 onto Costar Snapwell inserts (0.45-μM pore size, 1 cm^2 surface area) coated with type VI human placental collagen (Sigma, St. Louis, MO). For the first 6 days, cells were grown submerged in culture medium that was changed every 2-3 days. Subsequently, air interface culturing was used, in which the medium was added only to the basolateral side of the inserts. For whole-cell patch clamp studies, cells were seeded onto 35 mm plates (Becton Dickinson, Franklin Lakes, NJ), at a density of 1.5 x 10^3 cells/cm^2, at least four hours prior to experiments.

B) Whole-cell patch-clamp:

Pipette electrodes were made from thin-walled borosilicate glass (A-M Systems Inc., Everett, WA) using a two-stage vertical puller (Nirashige, Japan). Electrode tips were fire polished to a final resistance of 3-6 MΩ immediately before experiments. The composition of pipette and bath solutions is given in Table 2.1. Cultured cells were rinsed three times in bath solution immediately before being mounted into a holder fixed to the

stage of an Olympus IMT-2 Inverted Research Microscope (Lake Success, NY). The holder maintained the bath solution at 37°C by means of a heat-exchange perfusion system. After the pipette had been immersed in bath solution, offset potentials were compensated before forming a GΩ seal. Once sealed, the whole-cell configuration was obtained mechanically, by suction, and the cell was immediately clamped to –40 mV. Currents were recorded, at 1 min intervals, using an Axopatch 200A amplifier and Clampex 8.0 software, both from Axon Instruments (Foster City, CA), in response to the voltage protocol shown in Figure 2.1E. All currents were reported with reference to zero in the bath. The access resistance of the patch and cell capacitance was measured directly by the compensation circuitry of the patch clamp amplifier, as well as by Clampex 8.0 software. The whole-cell capacitance in these experiments, expressed as a mean ± S.D., was 27 ± 6 pF (n=125) and only seals with a series resistance of less than 20 MΩ were used. All data were analyzed by Clampfit 8.0 (Axon Instruments), Microsoft Excel 97 (Seattle, WA), and Micrococal Origin 5.0 (Northampton, MA) software. Traces were first normalized to 1 pF, in order to remove variability due to cell size. The current-voltage relationship was obtained from the mean current during the central 140 ms of the recording. The whole-cell current chord conductance, γ, was computed from the equation:

$$I = \gamma (V - E_{rev})$$

where I is the whole-cell current, V is the applied voltage, and E_{rev} is the whole-cell current reversal potential. Calculations of chord conductance were performed at V = 40 mV.

Data are presented as means ± SEM; n refers to the number of experiments. The

paired Student's t-test was used to compare the means of two groups. Statistically significant differences among the means of multiple groups were determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer post test using Graphpad Instat 3.05 software (San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

C) RT-PCR:

Total RNA was isolated from 2 x 10^6 cells using the Qiagen RNeasy kit (Qiagen). The average amount of RNA obtained from 2 x 10^6 cells was ~400 ng. One fourth of the RNA was reverse transcribed using superscript II reverse transcriptase (Gibco BRL) and either oligo(dT) or random hexamers ($50A_{260}$ units; Boehringer Mannheim) as primers. Thereafter, PCR was performed in 20 µl reactions using the primer pairs (25 µM) described in Table 2.2. In addition to the primers designed to amplify sequences of interest, reactions with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers were run in all rounds of PCR reactions to serve as internal positive controls. One tenth of the cDNA was used in PCR experiments, and amplification proceeded by annealing for 30 s at the temperatures indicated (Table 2.2), followed by an elongation step at 72° C for 1 min. Sequences were amplified over 30 or 38 cycles and PCR products with the expected sizes, shown in Table 2.2, were resolved on 1.5% agarose gels. All RT-PCR products were sequenced in one (adenosine receptors and IK-1 channels) or both (nucleoside transporters) directions by Taq dideoxyterminator cycle sequencing using an automated Model 373A DNA sequencer (Applied Biosystems, Foster City, CA).

D) Nucleoside transport:

Experiments were carried out at 20°C in HEPES-buffered Ringer's solution (HPBR) containing (in mM) 135 NaCl, 5.0 KCl, 3.33 NaH_2PO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 5.0 HEPES (pH = 7.4 at 20°C) or in Na^+-free HPBR containing (in mM) 140 N-methyl-D-glucamine (NMG), 5.0 KH_2PO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 5.0 HEPES (pH = 7.4 at 20°C). Confluent monolayers of A549 cells grown on permeable filters were washed six times with HPBR or Na^+-free HPBR and then incubated in the same solution (± 1 μM NBTI) for 30 min. Uptake was initiated by adding 10 μM ^{14}C-labelled adenosine or uridine (0.5 μCi/ml, Amersham Pharmacia Biotech) in HPBR or Na^+-free HPBR (± 1 μM NBTI) to either the apical or basolateral compartment. Incubations with adenosine also included 1 μM deoxycoformycin to inhibit adenosine deaminase activity. Uptake was terminated after 30 s to 3 min by ten rapid washes of the cell culture inserts in an ice cold "stop" solution containing (in mM) 100 $MgCl_2$ and 10 Tris-HCl (pH = 7.4 at 0°C) (32). The monolayers were dissolved in 0.2 ml 5% (w/v) SDS and counted for radioactivity using a Beckman LS 6000IC liquid scintillation counter (Irvine, CA). Non-mediated (passive) uptake was determined in the presence of 1 μM NBTI and excess (5 mM) unlabeled uridine. The protein content of representative monolayers was measured using the Bio-Rad Protein Standard Assay Procedure. The flux values shown are means ± SEM of n=5 inserts. Each experiment was repeated at least three times on different batches of cells.

E) Chemicals:

Adenosine and deoxycoformycin were prepared in H_2O as 10 mM and 1 mM stock solutions, respectively. NBTI was prepared as a 3 mM stock solution in methanol. clotrimazole as a 30 mM stock solution in ethanol, and amiloride as a 10 mM stock solution in H_2O. All the above drugs were obtained from Sigma. 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-15943) was prepared as a 2 mM stock solution in DMSO, 1-3-dipropyl-8-cyclopentylxanthine (DPCPX) was prepared as a 10 μM stock solution in 0.1 N NaOH, 3,7-dimethyl-1-propargylxanthine (DMPX) was prepared as a 5 mM stock solution in H_2O and all three were purchased from RBI (Natick, MA). Finally, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, Molecular Probes Inc. Eugene, OR) was prepared as a 5 mM stock solution in H_2O, 1-ethyl-2-benzimidazalinone (1-EBIO, Aldrich, Milwaukee, WI) as a 600 mM stock solution in ethanol, and 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone (XE991, a generous gift from Dr. B.S. Brown, DuPont, Wilmington, DE) as a 10 mM stock solution in 0.1 N HCl.

RESULTS

A) The effect of exogenous and autocrine adenosine on whole-cell current in A549 cells:

Figure 2.1A shows typical recordings of whole-cell current in A549 cells obtained in nonsymmetrical cationic solutions (pipette 135 mM KCl, bath 135 mM NaCl). Addition of adenosine (100 μM) to the bath solution significantly activated whole-cell current ($n=8$, $P < 0.01$). The effect of autocrine adenosine on whole-cell current was studied using NBTI, an inhibitor of equilibrative nucleoside transporters. We reasoned that if the uptake of extracellularly produced adenosine is inhibited, then its concentration would increase, leading to activation of adenosine receptors. Figure 2.1B shows that the addition of NBTI (10 μM) to the bath solution had an effect on the whole-cell current that was similar to the addition of exogenous adenosine ($n=15$, $P < 0.01$). Similar experiments performed with NBTI at a concentration that specifically inhibits hENT1 but not hENT2 (1 μM) showed that there was no significant difference ($P > 0.05$, $n=6$) in whole cell current activation by these two concentrations of inhibitor (data not shown). These results suggested that hENT1 may mediate the majority of nucleoside transport in A549 cells.

The observation that NBTI had no effect on the whole-cell current in the presence of exogenous adenosine suggests a common mechanism of current activation by action on adenosine receptors (Fig. 2.2). This conclusion was further supported by experiments showing that addition of the nonselective adenosine receptor antagonist, CGS-15943 (1 μM), reversed activation of the whole-cell current by NBTI (Fig. 2.3). Interestingly,

CGS-15943 alone had no effect on the basal whole-cell current ($P > 0.05$, n=6, data not shown).

Figure 2.4 shows the effects of adenosine receptor antagonists on the whole-cell current. DPCPX, a specific antagonist of A_1 receptors, reversed the effect of adenosine, indicating that these receptors are involved in current activation. In contrast, A_{2A} and A_{2B} receptors appeared not to be involved in this process, since their antagonist, DMPX, had no effect on the current activated by adenosine (Fig. 2.4B,D).

B) Identification of channel types activated by adenosine:

To determine the channel types involved in the response to adenosine, ion replacement and pharmacological studies were performed. Replacement of K^+ ions in the pipette and bath solutions by Cs^+ reduced basal conductance by 60%, indicating that K^+ channels contribute to basal whole-cell current (n=3, $P < 0.05$). Under these conditions, addition of adenosine to the bath solution had no effect on the whole-cell current, indicating that K^+ channels are the major targets for adenosine action (n=3, $P > 0.05$).

Epithelial cells possess two distinct classes of K^+ channels, regulated by cAMP- and Ca^{2+}-mediated agonists, respectively. Therefore, we used selective modulators of these channel families to characterize their contribution to the baseline and adenosine-stimulated current. A specific inhibitor of cAMP-dependent K^+ channels, XE991 (10 μM), appeared to affect neither basal whole-cell current nor the subsequent response to 100 μM adenosine, suggesting that they do not contribute to the cell membrane conductance (n=4, $P > 0.05$, paired Student's *t-test*). In contrast, an opener of intermediate conductance Ca^{2+}-dependent K^+ (IK) channels, 1-EBIO (600 μM), increased

the mean cell membrane conductance from 75 ± 5 pS/pF to 468 ± 35 pS/pF, and caused a shift in the reversal potential from –14 ± 3 mV to –68 ± 2 mV, indicating that these channels make a major contribution to the membrane conductance (n=4, $P < 0.0001$). This result was further supported by the use of clotrimazole, a selective inhibitor of IK channels (Fig. 2.5). Clotrimazole (10 μM) inhibited basal whole-cell current ($P < 0.05$, n=8), and abolished the subsequent response to adenosine, indicating that IK channels were a likely target for adenosine action.

The effect of adenosine on the activity of Na^+ and Cl^- channels was evaluated using amiloride and DIDS, respectively. Amiloride (10 μM), a specific blocker of epithelial Na^+ channels, appeared to affect neither basal whole-cell current nor the subsequent response to 100 μM adenosine (Fig. 2.6). In contrast, addition of 50 μM DIDS to the bath solution reduced the whole-cell current, indicating that DIDS-sensitive Cl^- channels make a contribution to the basal current (Fig. 2.7). However, adenosine in the presence of DIDS significantly increased the current, indicating that other channels were activated by adenosine. In summary, these results have demonstrated that stimulation of A_1 receptors, in A549 cells, activates K^+ transport, likely through IK channels.

C) Identification of adenosine receptors, nucleoside transporters, and IK channels using RT-PCR:

The regulatory actions of adenosine are mediated via four subtypes of G-protein coupled receptors distinguished as A_1, A_{2A}, A_{2B} and A_3. Gene expression of these receptors was investigated using RT-PCR. As shown in Figure 2.8A, A549 cells express mRNA for A_1, A_{2A} and A_{2B} but not A_3 receptors. Interestingly, studies with selective adenosine receptor

antagonists indicate that only the A_1 receptor is involved in the regulation of whole-cell current.

Electrophysiological studies indicated that IK channels function in A549 cells, and are involved in the response to adenosine. Our RT-PCR data confirm the presence of mRNA for the IK-1 protein in A549 cells (Fig. 2.8B).

Figure 2.8C shows RT-PCR amplification of nucleoside transporter transcripts in A549 cells. The cells contained mRNA for both equilibrative nucleoside transporters (hENT1 and hENT2), but lacked transcripts for concentrative nucleoside transporters (hCNT1, hCNT2, and hCNT3).

All PCR products were sequenced and found to be identical to corresponding GeneBank™ sequences (accession numbers given in Table 2.2). Control amplifications in the absence of added A549 cDNA were negative for all successfully identified adenosine receptors (A_1, A_{2A} and A_{2B}), IK channels (IK-1) and nucleoside transporters (hENT1 and hENT2), while control amplifications done with GAPDH primers were always positive.

D) Functional studies of nucleoside transport by A549 cells:

Apical and basolateral uptake of adenosine was measured as a function of time at room temperature and, as shown in Figure 2.9, was linear for 3 min (apical > basolateral). Subsequent initial rate measurements, to determine the basolateral and apical pathways for adenosine and uridine transport were carried out using a 2 min incubation and are shown in Figure 2.10. Apical transport of adenosine was not significantly reduced by removal of extracellular Na^+, but was substantially inhibited by 1 μM NBTI, a

concentration sufficient to block all hENT1-mediated transport activity (Fig. 2.10A). NBTI-insensitive adenosine uptake was reduced further in the presence of excess unlabelled uridine. Since adenosine and uridine are both transported by hENT1 and hENT2, this result identifies additional hENT2-mediated and passive components of adenosine uptake. At the concentration of adenosine tested (10 μM), the ratio of the contribution of hENT1 (NBTI-sensitive) relative to hENT2 (NBTI-insensitive), corrected for passive uptake, was 9:1. Basolateral adenosine transport (Fig. 2.10B) as well as apical and basolateral transport of uridine (Figs. 2.10C, D), a universal ENT/CNT permeant, showed similar characteristics (hENT1:hENT2 flux ratios 12:1, 5:1 and 4:1, respectively). These results were consistent with the electrophysiological data, which indicated that the majority of nucleoside transport that effects whole-cell current is mediated by hENT1.

DISCUSSION

Extracellular adenosine has been previously shown to modulate the function of both cation (3,6,29,39) and anion (5,6,10,26,33,39) channels in several epithelia. There are also reports suggesting that the presence of nucleoside transporters in epithelial cells may regulate these effects by controlling the effective concentration of adenosine in the vicinity of its receptors (28,32). The results presented in this paper confirm and extend these observations by identifying and characterizing the ion channels, adenosine receptors and nucleoside transporters involved in the regulation of whole-cell current in A549 cells.

In the lung, most of the extracellular adenosine is derived from cleavage of the nucleotide adenosine 5'-monophosphate (AMP) by the enzyme 5'-nucleotidase, which is located on the outer surface of the cell plasma membrane (35). AMP in turn, may be generated from epithelium-derived extracellular ATP and other adenosine nucleotides, including cAMP. Since ATP is present at mM concentrations in the cytoplasm, it is possible that release of ATP from injured airway cells contributes to the increased concentration of adenosine found in the airway surface liquid of asthmatic patients. In addition, it has been suggested that cells may secrete ATP, by Ca^{2+}-dependent vesicular exocytosis, or through ATP transporters whose identity is still controversial (22). Intracellular levels of adenosine are normally kept low mainly by its conversion to AMP by the enzyme adenosine kinase, which creates an inwardly directed gradient for adenosine entry into the cell. However, the possibility of direct adenosine release from the airway epithelium cannot be excluded, particularly under conditions of stress.

The results presented in this study show that application of NBTI, a selective inhibitor of hENT1-mediated adenosine transport, had similar effects on whole-cell current as the application of exogenous adenosine. Furthermore, the effect of NBTI was not additive with that of adenosine, and was inhibitable by the adenosine receptor antagonist, CGS-15943, indicating that NBTI effect is mediated through the activation of adenosine receptors. Therefore, adenosine transporters could regulate epithelial electrolyte secretion by controlling adenosine concentration in the vicinity of its receptors.

It is important to note that CGS-15943 alone had no effect on the baseline current, indicating that endogenous adenosine does not affect the baseline whole-cell current. However, several observations suggest that the effect of autocrine adenosine *in vivo* may be different from that *in vitro*. First, epithelial cells are normally covered by a thin (~10 μm) layer of airway surface liquid *in vivo*, while cells in our experiments were covered by a ~1 cm thick layer of bath solution. Second, *in vivo* the whole epithelial monolayer contributes to adenosine generation, in contrast to a single cell in patch clamp studies. Third, other cell types (e.g. mast cells) in the vicinity of the epithelium may contribute to extracellular adenosine concentration *in vivo*.

The regulatory actions of adenosine are mediated via four subtypes of G-protein coupled receptors, distinguished as A_1, A_{2A}, A_{2B} and A_3 (17,25). Activation of each of these receptors has been linked with the regulation of ion transport in epithelial tissues (3,5,26,39). The results of RT-PCR experiments have shown that A549 cells express A_1, A_{2A}, and A_{2B} but not A_3 receptors. However, functional studies with specific adenosine receptor antagonists indicate that only the A_1 receptor is involved in the regulation of whole-cell current by adenosine. Since A_1 receptors are linked to $G_{i1/2/3}$ proteins, and

their activation increases IP_3 generation and $[Ca^{2+}]_i$ (17), it is likely that adenosine stimulates whole-cell current by activation of Ca^{2+}-dependent ion channels.

Adenosine receptor activation has been shown to activate CFTR Cl^- channels (11), non-CFTR Cl^- channels (4,33), amiloride-sensitive Na^+ channels (3,29) and Ca^{2+}-dependent K^+ channels (39). Since A549 cells do not express CFTR (16), they constitute a convenient model for the study of the regulation of non-CFTR anion channels. DIDS reduced whole-cell current in A549 cells, indicating significant contribution of non-CFTR anion channels to the basal whole-cell current. Interestingly, subsequent application of adenosine activated whole-cell current indicating that DIDS-sensitive anion channels may not be targeted by adenosine.

A549 cells have been recently shown to contain amiloride-sensitive Na^+ channels with molecular and biophysical properties similar to those of alveolar type II cells (27). However, the data from the present study showed whole-cell current activation in the presence of amiloride, suggesting that amiloride-sensitive Na^+ channels are not affected by adenosine. Similar to other examples of tissue-specific regulation of Na^+ channels (for a review see 31), this result is clearly different from the effect of adenosine on amiloride-sensitive Na^+ channels in the kidney (29) and intestine (3), where adenosine has been shown to be a potent regulator of their function.

Ion replacement studies demonstrated that K^+ ions make a major contribution to the basal whole-cell current in A549 cells. Studies from several laboratories have shown that Ca^{2+}- and cAMP-mediated agonists regulate IK and KCNQ channels, respectively. IK channels, which are apparently absent in excitable tissues, are predominantly expressed in peripheral tissues including endothelia, epithelia and the hematopoietic system (23,24).

These channels are thought to play a crucial role in the regulation of Cl^- and HCO^-_3 secretion in human airway epithelial cells (13). Activation of basolateral cAMP-dependent K^+ channel, K_vLQT1 (KCNQ1), in parallel with the apically located CFTR, has been shown to play an important role in maintaining cAMP-dependent Cl^- secretion in human airways (30). In this study we found that XE991, a specific inhibitor of cAMP-dependent K^+ channels (40), had no effect on basal whole-cell current or the subsequent response to 100 μM adenosine, suggesting a lack of their contribution to the cell membrane conductance. In contrast, studies with an opener (1-EBIO) and a blocker (clotrimazole) of IK channels showed that these are a major contributor to baseline current. Similarly, the fact that clotrimazole abolished the current response to adenosine indicated that IK channels were a target for adenosine action.

Epithelial nucleoside transport has been most extensively studied in intestine, kidney, liver and choroid plexus (1,7,46). Enterocytes of the small intestine, for example, contain transcripts for all five of the CNT and ENT transporter isoforms (21,36,37,44,45) and express CNT1/2 functional activity in their apical membrane and ENT1 and/or ENT2 functional activity at the basolateral membrane (reviewed in 46). Cultured T84 cells, a model of intestinal crypt cells, express basolaterally-restricted ENT1/2 functional activity and nucleoside uptake across the apical membrane having the characteristics of passive diffusion (32,42). Similar properties have been described for the colonic epithelial cell line Caco-2 (42), although an earlier study found these cells to express CNT3-type transport activity at the apical surface (2). Immunocytochemical analyses have demonstrated the presence of CNT1 protein in the apical membrane of rat small intestine, but not at the basolateral membrane, and a similar apical localization was identified for

kidney proximal tubule (20). In rat liver parenchymal cells, CNT1 was abundant in bile canalicular membranes, but largely excluded from sinusoidal membranes (20) which, instead, are enriched in CNT2 immunoreactivity (15). Choroid plexus expresses CNT3-type functional activity (43). Much less is known about nucleoside transport in other epithelia, although pharmacological and RT-PCR studies suggest the presence of CNT2 but not CNT1 in rat epididymal epithelium (9).

In the present study, we have used complementary molecular and functional approaches to investigate the nucleoside transport capabilities of A549 cells. In the first series of experiments, RT-PCR was used in conjunction with isoform-specific oligonucleotide primers to test for the presence of hCNT1, hCNT2, hCNT3, hENT1 and hENT2 mRNA. In the second, transport studies were used to confirm the identity of the expressed nucleoside transporters, and to investigate their vectorial distribution (apical versus basolateral membrane). Our results show that A549 cells lack transcripts for hCNT1, hCNT2 and hCNT3. Functionally, we also failed to detect any Na^+-dependent adenosine or uridine transport activity. Thus, A549 cells represent another example of an epithelial cell line lacking Na^+-dependent mechanisms of nucleoside transport. Instead, RT-PCR analyses identified transcripts for hENT1 and hENT2. Both transport activities were detected in apical as well as basolateral membranes (hENT1 > hENT2). While hENT1 and hENT2 both transport adenosine and uridine and are broadly selective for other purine and pyrimidine nucleosides, the two transporters are not functionally equivalent. For example, hENT1 has generally higher apparent substrate affinities, while hENT2 is also capable of interacting with nucleobases (12,18,19). The two transporters may therefore fulfill complementary, but distinct physiological functions.

In summary, the results of this study show that both adenosine receptors and transporters control adenosine effects on K^+ channel function in A549 cells. Extracellularly generated adenosine is either transported via ENT1 (or to a lesser extent ENT2) into the cell, or can activate adenosine receptors expressed on the cell surface. Inhibition of adenosine transport leads to an increase in adenosine concentration in the extracellular space and activation of adenosine receptors. Nucleoside transport may therefore represent an endogenous regulatory mechanism for adenosine-dependent control of ion secretion in human airway epithelial cells. A better understanding of this system could lead to the development of a novel therapeutic strategy in asthma and other respiratory disorders characterized by altered composition and quantity of airway surface liquid.

ACKNOWLEDGMENTS

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TABLES

Table 2.1

Patch clamp solutions

	Standard Pipette solution (mM)	Standard Bath solution (mM)	K^+ Free Pipette solution (mM)	K^+ Free Bath solution (mM)
NaCl	5	135	5	135
KCl	135	5	-	-
$MgCl_2$	1.8	1	1.8	1
$CaCl_2$	0.2	1	0.2	1
Glucose	10	10	10	10
HEPES	10	10.5	10	10.5
EGTA	0.5	-	0.5	-
CsCl	-	-	135	5

pH = 7.4 at 37°C

Table 2.2

Summary of RT-PCR Primers and Conditions

mRNA	Primer Positions (5' to 3')	PCR Product Size (bp)	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Cycles
GAPDH	212-235 806-786	595	cca ccc atg gca aat tcc atg gca tca aga cgg cag gtc agg tcc acc	60	30
A_1	603 - 623 847 - 826	245	ctc gcc atc ctc atc aac att cag cca aac ata ggg gtc agt c	55	30
A_{2A}	546-569 995-972	450	gcc cct ctc tgg ctc atg tac ctg tca tca gga cac tcc tgc tcc atc	55	30
A_{2B}	447 - 466 958 - 938	512	cag acg ccc acc aac tac tt gcc acc agg aag atc tta atg	55	30
A_3	353-376 793-770	441	aac gtg ctg gtc atc tgc gtg gtc gta gtc cat tct cat gac gga aac	55	30
hENT1	623 - 644 950 - 930	327	ctc att aat tca ttt ggt gcc a cct ctc ctt tgc taa tga ggt	58	38
hENT2	410 - 430 751 - 731	341	tca tca act cct tca gtg cag gaa tcc cgt tct cat cag act	58	38
hCNT1	1 - 23 612 - 593	613	tgg aag gtc tgg gac atg gag aa atg atg ctt tga gca ggc aa	58	38
hCNT2	1-22 540-521	541	gag gag aac agg aga tgg aga a gga tca gct gct ctg gcc tt	58	38
hCNT3	864 - 887 1319 - 1295	456	aga caa gtt cag act ttt ctg gag agg cca aaa gag ttt agc agc agc c	56	30
IK-1	1099-1118 1289-1270	191	ggg cac ctt tca gac aca ct acg tgc ttc tct gcc ttg tt	56	30

GenBank™ accession numbers: GAPDH, M33197; A_1, L22214; A_{2A}, U40771; A_{2B} X68487; A_3, L22607; hENT1, U81375; hENT2, AF029358; hCNT1, U62968; hCNT2, AF036109; hCNT3, AF305210; IK-1, AF022797.

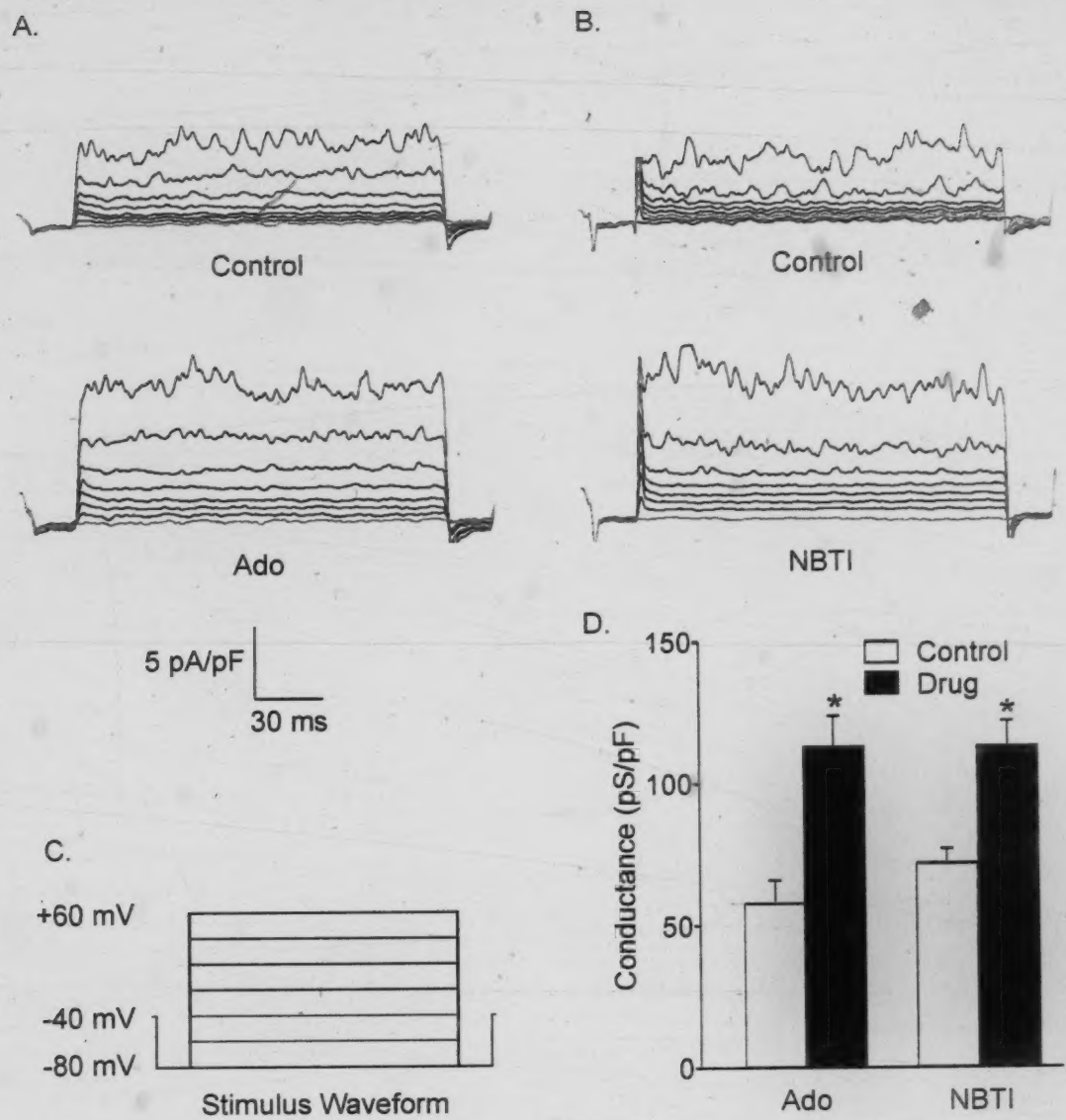
A.
B.
Control
Control
Ado
NBTI
5 pA/pF
30 ms
C.
+60 mV
-40 mV
-80 mV
Stimulus Waveform
D.
Control
Drug
Conductance (pS/pF)
150
100
50
0
*
*
Ado
NBTI

Figure 2.1

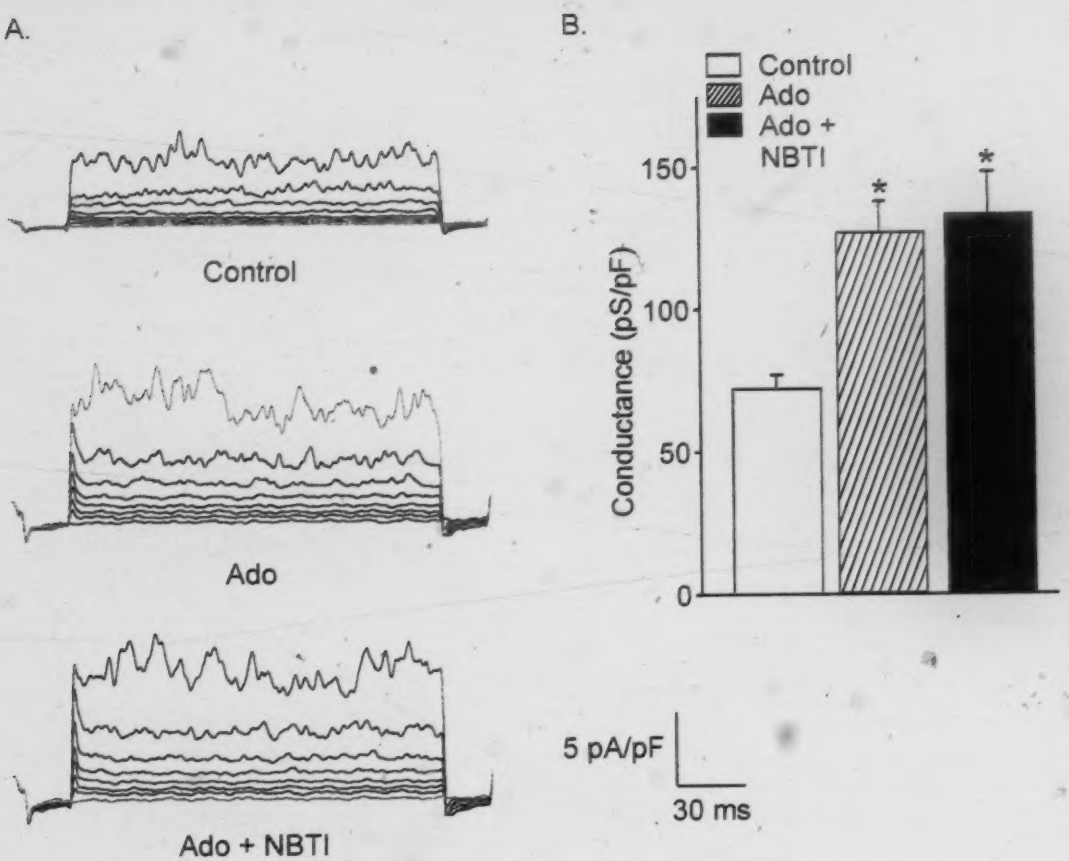
A.
Control
Ado
Ado + NBTI
B.
Control
Ado
Ado +
NBTI
150
100
50
0
Conductance (pS/pF)
*
*
5 pA/pF
30 ms

Figure 2.2

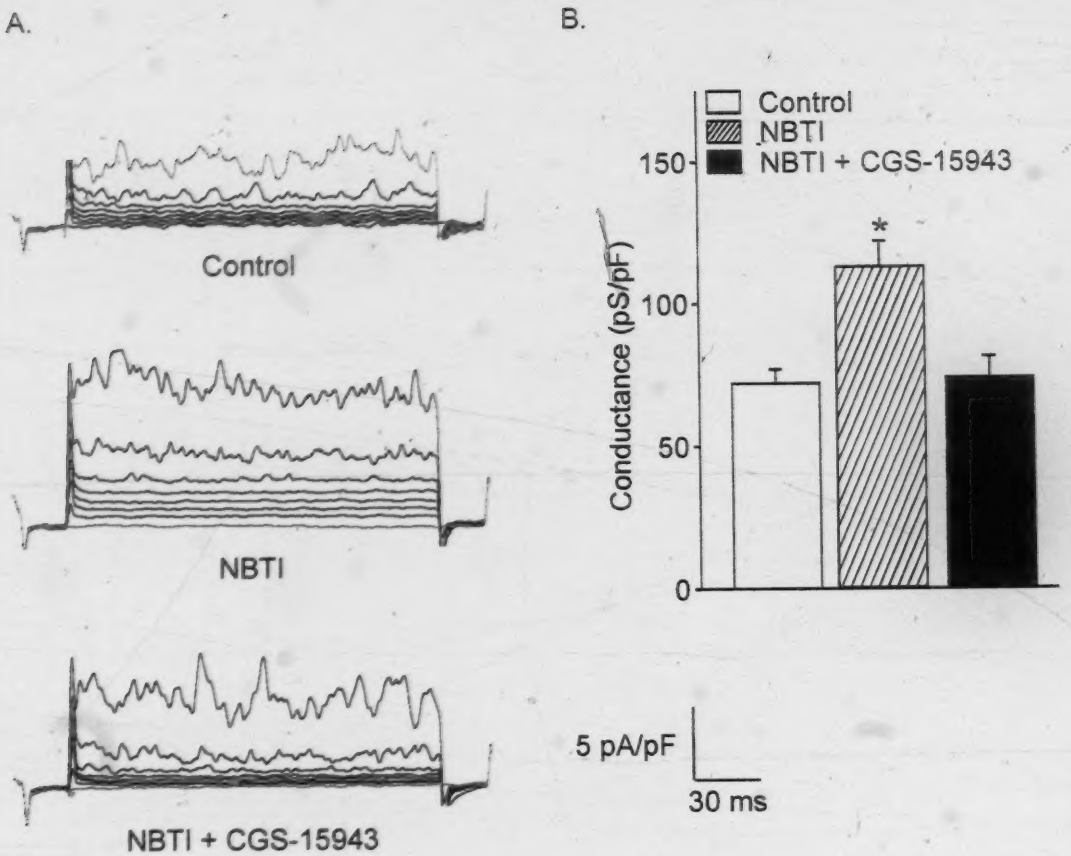
A.
Control
NBTI
NBTI + CGS-15943
B.
Control
NBTI
NBTI + CGS-15943
Conductance (pS/pF)
150
100
50
0
*
5 pA/pF
30 ms

Figure 2.3

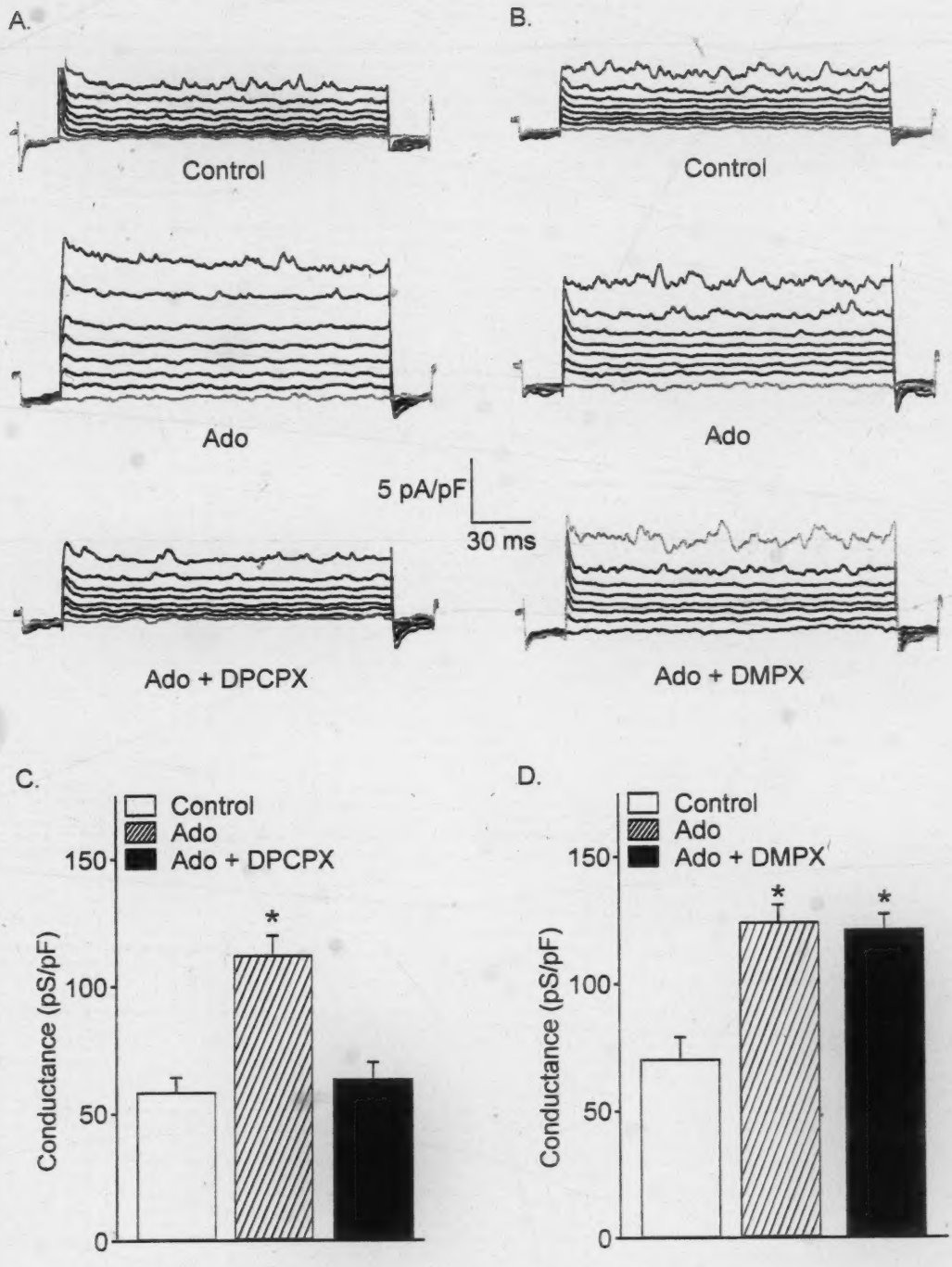
A.
Control
Ado
Ado + DPCPX
B.
Control
Ado
Ado + DMPX
5 pA/pF
30 ms
C.
Control
Ado
Ado + DPCPX
Conductance (pS/pF)
150
100
50
0
*
D.
Control
Ado
Ado + DMPX
Conductance (pS/pF)
150
100
50
0
*
*

Figure 2.4

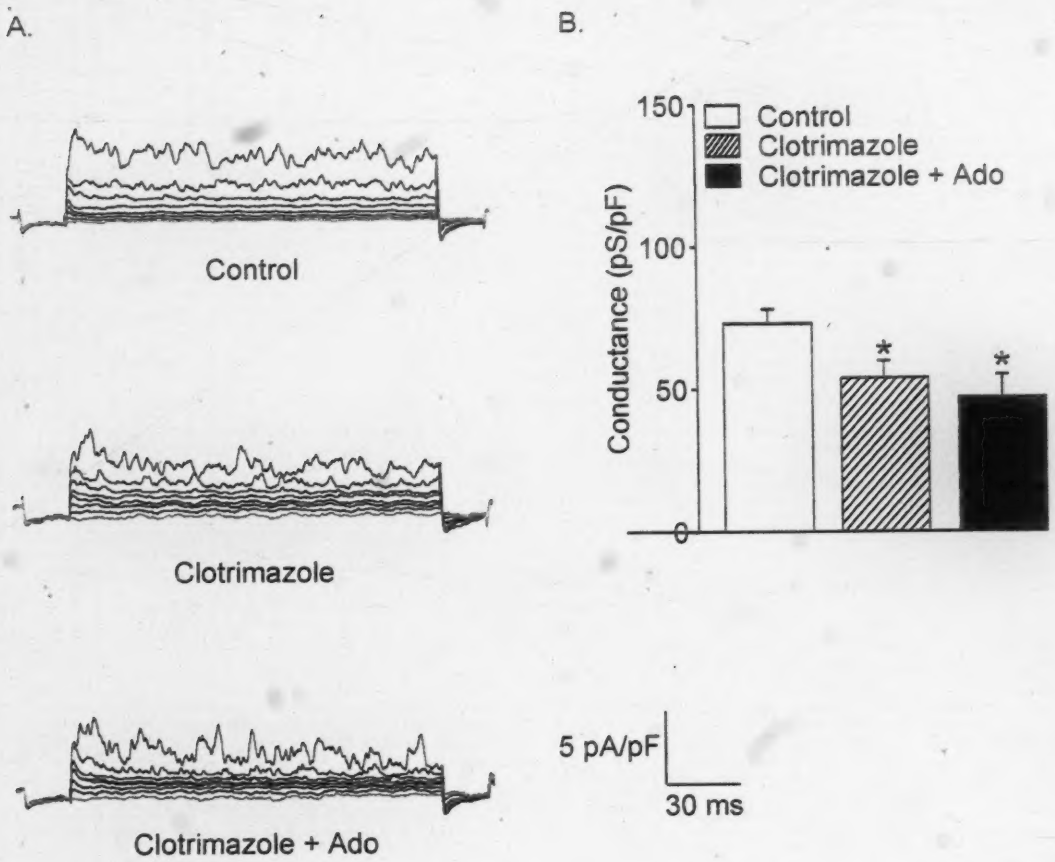
A.
B.
Control
Clotrimazole
Clotrimazole + Ado
150
100
50
0
Conductance (pS/pF)
Control
Clotrimazole
Clotrimazole + Ado
*
*
5 pA/pF
30 ms

Figure 2.5

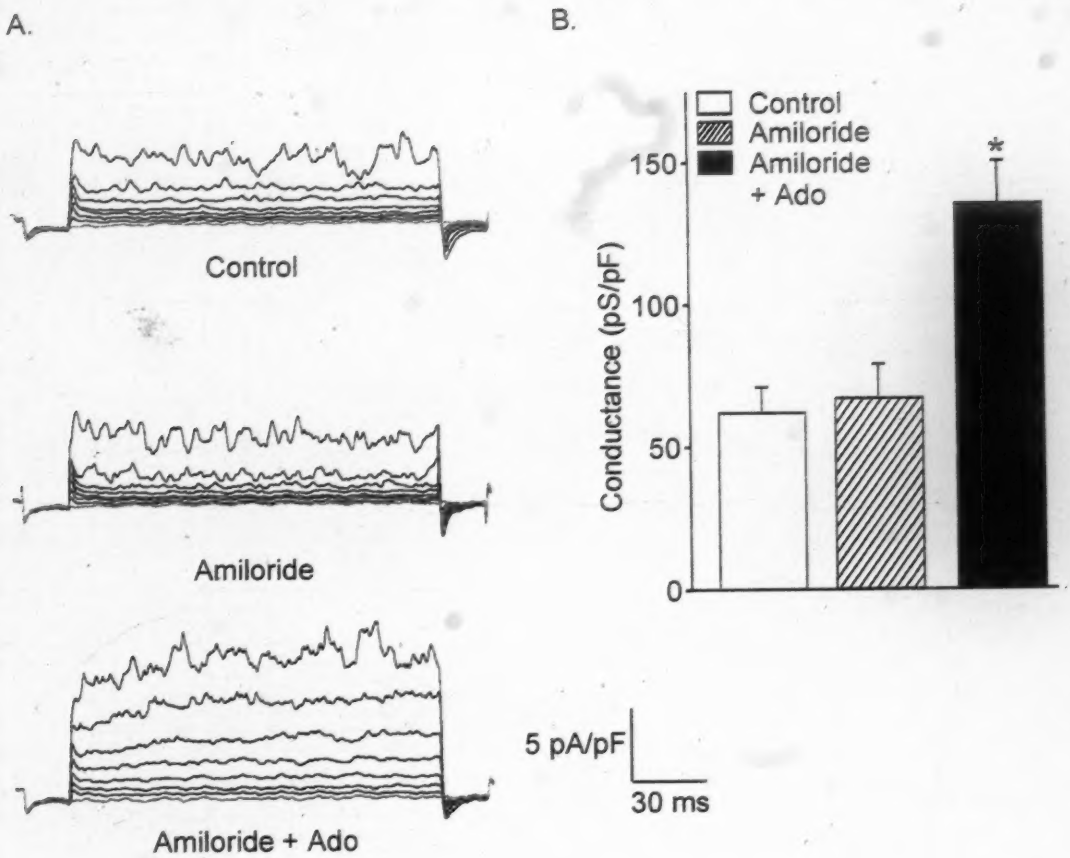
A.
B.
Control
Amiloride
Amiloride + Ado
Control
Amiloride
Amiloride
+ Ado
150
100
50
0
Conductance (pS/pF)
*
5 pA/pF
30 ms

Figure 2.6

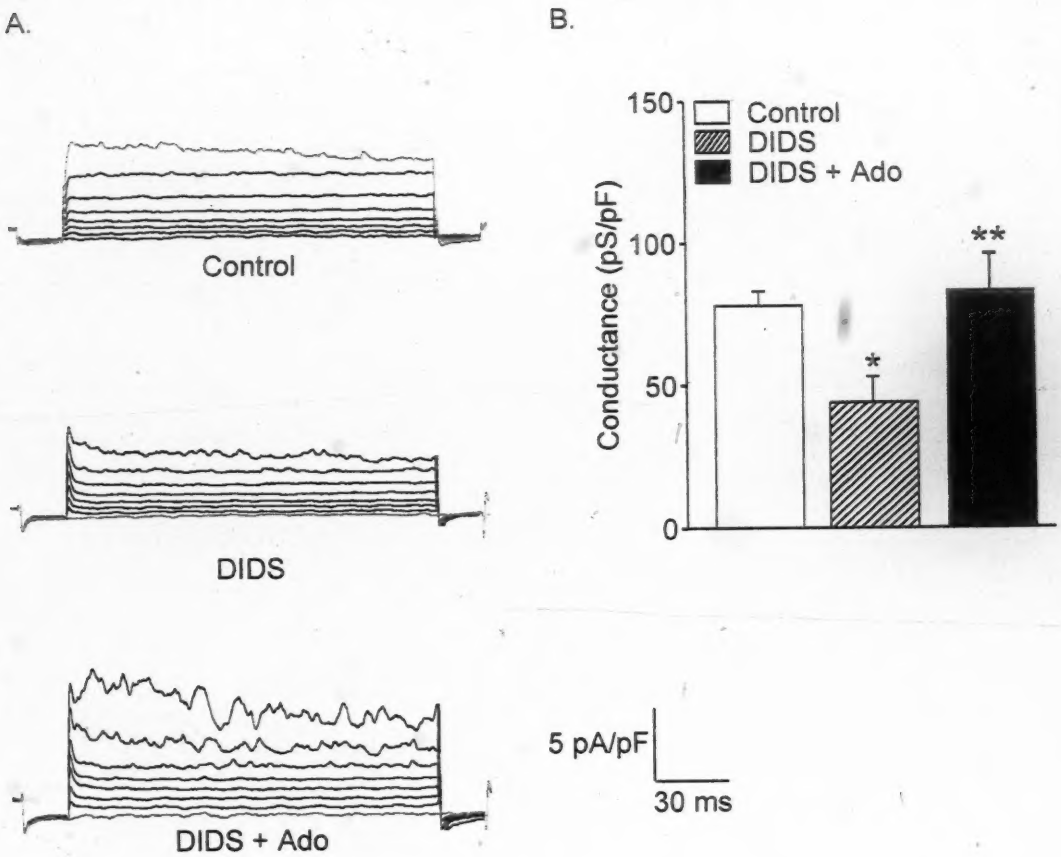
A.
B.
Control
DIDS
DIDS + Ado
150
100
50
0
Conductance (pS/pF)
Control
DIDS
DIDS + Ado
*
**
5 pA/pF
30 ms

Figure 2.7

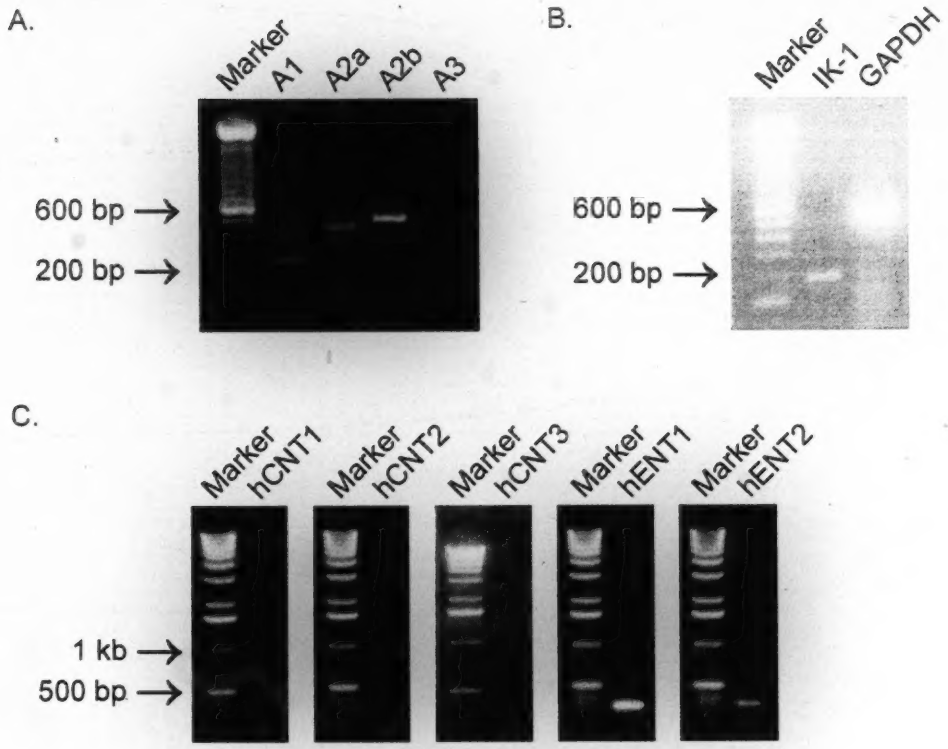
A.
Marker
A1
A2a
A2b
A3
600 bp
200 bp
B.
Marker
IK-1
GAPDH
600 bp
200 bp
C.
Marker
hCNT1
Marker
hCNT2
Marker
hCNT3
Marker
hENT1
Marker
hENT2
1 kb
500 bp

Figure 2.8

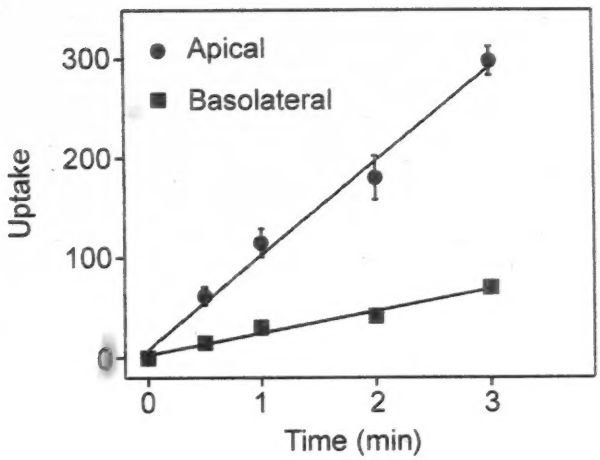

Figure 2.9

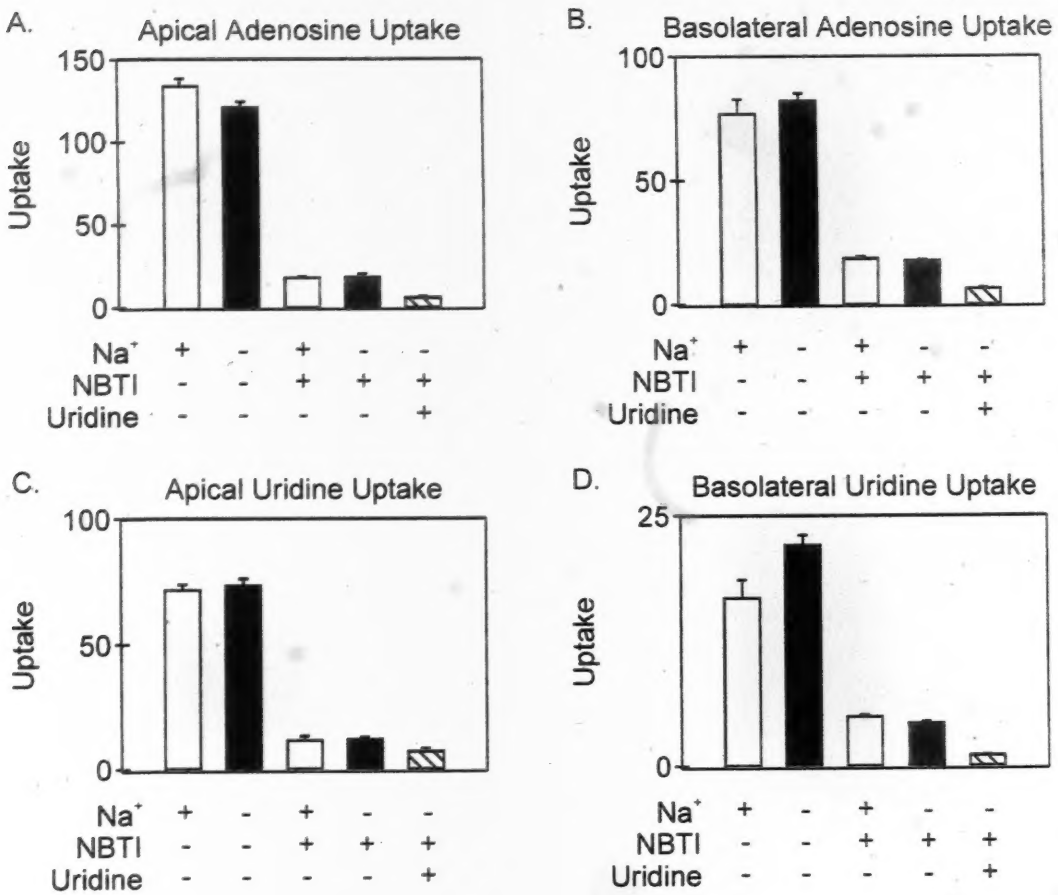
A.
Apical Adenosine Uptake
Uptake
150
100
50
0
Na+ + - + - -
NBTI - - + + +
Uridine - - - - +
B.
Basolateral Adenosine Uptake
Uptake
100
50
0
Na+ + - + - -
NBTI - - + + +
Uridine - - - - +
C.
Apical Uridine Uptake
Uptake
100
50
0
Na+ + - + - -
NBTI - - + + +
Uridine - - - - +
D.
Basolateral Uridine Uptake
Uptake
25
0
Na+ + - + - -
NBTI - - + + +
Uridine - - - - +

Figure 2.10

FIGURE LEGENDS

Figure 2.1

Stimulatory effect of adenosine and NBTI on whole-cell current. ***A:*** Representative traces showing the activation of whole-cell current by 100 μM adenosine (Ado, n=8). ***B:*** Representative traces showing the activation of whole cell current by 10 μM NBTI (n=15). ***C:*** Voltage protocol used for all experiments performed in this study. ***D:*** Summary of experiments done with adenosine and NBTI showing the change in membrane chord conductance, upon addition of nucleoside or nucleoside transport inhibitor. All values are expressed as mean chord conductance density (pS/pF) ± SEM. *Indicates statistical significance ($P < 0.01$).

Figure 2.2

Adenosine and NBTI, acting in combination, display no additive effects on whole-cell current, suggesting a common pathway of activation. ***A:*** Representative traces showing no further activation of whole-cell current when NBTI (10 μM) is added to cells treated with adenosine (Ado, 100 μM, n=3). ***B:*** Summary of experiments done with adenosine in combination with NBTI. All values are expressed as mean chord conductance density (pS/pF) ± SEM. * $P < 0.05$.

Figure 2.3

CGS-15943 inhibits NBTI-stimulated whole-cell current. ***A:*** Representative traces showing inhibition of 10 μM NBTI-stimulated whole-cell current with 1 μM CGS-15943

(n=6). ***B***: Summary of experiments done with NBTI followed by CGS-15943. All values are expressed as mean chord conductance density (pS/pF) ± SEM. * $P < 0.05$.

Figure 2.4

DPCPX, but not DMPX, inhibits adenosine stimulated current. ***A***: Representative traces showing inhibition of 100 μM adenosine stimulated whole-cell current with 10 nM DPCPX (n=6). ***B***: Representative traces showing that 10 μM DMPX does not inhibit adenosine stimulated whole-cell current (n=6). ***C***: Summary of experiments performed with adenosine followed by DPCPX. ***D***: Summary of experiments performed with adenosine followed by DMPX. All values in panels ***C*** and ***D*** are expressed as mean chord conductance density (pS/pF) ± SEM. * $P < 0.05$.

Figure 2.5

Clotrimazole inhibits whole-cell current and the subsequent response to adenosine. ***A***: Representative traces showing clotrimazole (10 μM) inhibition of whole-cell current (n=8) and subsequent lack of response to 100 μM adenosine (Ado, n=3). ***B***: Summary of experiments showing the effect of clotrimazole alone and in combination with adenosine. Values are expressed as mean chord conductance density (pS/pF) ± SEM. * $P < 0.05$.

Figure 2.6

Amiloride effects neither basal whole-cell current nor the subsequent response to adenosine. ***A***: Representative traces showing no effect of amiloride (10 μM, n=6) on whole-cell current, as well as no subsequent alteration of response to 100 μM adenosine

(Ado, n=4). ***B:*** Summary of experiments showing the effect of amiloride alone and in combination with adenosine on cell membrane chord conductance density (pS/pF), expressed as mean ± SEM. * $P < 0.05$.

Figure 2.7

DIDS inhibits basal whole-cell current but does not prevent the subsequent response to adenosine. ***A:*** Representative traces showing DIDS (50 μM) inhibition of whole-cell current (n=12) and subsequent response to 100 μM adenosine (Ado, n=12). ***B:*** Summary of experiments showing the effect of DIDS alone when compared to baseline (*$P < 0.05$), and in combination with adenosine when compared to DIDS alone (** $P < 0.05$). Values are expressed as mean chord conductance density (pS/pF) ± SEM.

Figure 2.8

Expression of adenosine receptors, IK channels and nucleoside transporters, characterized by RT-PCR. ***A:*** A549 cells express transcripts for A_1, A_{2A} and A_{2B} but not A_3 receptors. ***B:*** A549 cells express IK-1 channel mRNA. A representative positive control, using primers specific for GAPDH mRNA, is also included. ***C:*** A549 cells express transcripts for both hENT1 and hENT2, but not for the hCNT1, hCNT2 or hCNT3 nucleoside transporters.

Figure 2.9

Time course of adenosine uptake across the apical and basolateral membranes of A549 cells. Uptake of 10 μM adenosine [pmol/(mg protein)] is greater across the apical

membrane than the basolateral membrane and it is linear across both membranes for the first 3 min. The data are presented as means ± SEM.

Figure 2.10

Functional characterization of nucleoside uptake in A549 cells. Initial rates of 10 μM adenosine and uridine uptake [pmol/(mg protein•min)] across the apical (***A***, ***C***) and basolateral (***B***, ***D***) membranes were measured over a 2 min time course. In all experiments, nucleoside uptake was not significantly effected by the removal of Na^+, indicating an absence of concentrative Na^+/nucleoside co-transport. Adenosine and uridine uptake across both apical and basolateral membranes was sensitive to inhibition by 1 μM NBTI. However, a small component of nucleoside uptake was not inhibited by NBTI but was sensitive to inhibition by excess unlabeled uridine (5 mM), indicating a minor contribution of NBTI-insensitive nucleoside transport to total nucleoside transport. The remaining nucleoside uptake was via passive (non-transporter mediated) mechanisms. Similar patterns were observed for adenosine and uridine uptake, indicating broad permeant selectivity for both NBTI-sensitive and NBTI insensitive mediated transport in A549 cells.

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CHAPTER 3

Coupling of CFTR-mediated anion secretion to nucleoside transporters and adenosine homeostasis in Calu-3 cells

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INTRODUCTION

Adenosine is an endogenous nucleoside that regulates physiological functions through G protein-coupled P_1 receptors. Four receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3, have been identified based on their molecular structure, pharmacology, and mechanisms of G protein-mediated signaling (8,17). Activation of each of these receptors has been linked to the regulation of ion transport in epithelial tissues (1,4). In airway epithelial cells, stimulation of A_{2B} receptors has been shown to activate anion conduction via a CFTR-dependent pathway (4,12), although the underlying mechanisms are not fully understood.

The magnitude of the effects of adenosine on ion transport is related to its concentration in the vicinity of its cell-surface receptors, which depends on the relative rates of its synthesis and metabolism (6). Extracellular adenosine is produced by hydrolysis of 5'-AMP by membrane-bound ecto-5'-nucleotidase; the 5'-AMP is itself produced by the action of nonspecific (alkaline or acidic) extracellular phosphatases on ADP and ATP. In addition, adenosine may also be produced from S-adenosylhomocysteine by the action of S-adenosylhomocysteine hydrolase. However, this pathway does not seem to play a major role in total adenosine production (22). Metabolism of adenosine can occur by the action of either adenosine kinase or adenosine deaminase (ADA), resulting in the conversion of adenosine to AMP or inosine, respectively (6). The balance in the rates of these activities will determine the amount of physiologically relevant adenosine that is present.

Given that the majority of adenosine production occurs extracellularly, while most of its metabolism occurs intracellularly, transport of adenosine across the plasma membrane may also be an important determinant of its concentration at cell surface receptors (2). Since adenosine is a hydrophilic molecule, it requires specialized transporter proteins for permeation of cell membranes. Two distinct families of nucleoside transporters have been characterized in mammalian cells, the equilibrative (Na^+-independent) nucleoside transporters (ENTs) and the concentrative (Na^+-dependent) nucleoside transporters (CNTs) (2). The ENTs are facilitative carriers that transport nucleosides down their normally inwardly directed concentration gradients, whereas the CNTs are secondary active symporters that use Na^+ gradients to transport nucleosides into cells. Molecular cloning has identified two functional members of the human ENT family. Both are ubiquitously distributed, but only hENT1 is sensitive to inhibition by nitrobenzylmercaptopurine ribonucleoside (NBMPR, K_i 0.1-10 nM), whereas hENT2 is unaffected by concentrations of NBMPR $\leq$ 1 μM. Furthermore, both transporter types display broad selectivities, accepting a structurally diverse group of pyrimidine and purine nucleosides as permeants. However, only hENT2 also transports nucleobases (25). The concentrative transporters have limited tissue distributions and have primarily been described in specialized cells, such as intestinal epithelia, renal epithelia, liver, choroid plexus, splenocytes, macrophages and leukemic cells (2,26). Three human members of this family have been identified by molecular cloning and characterized functionally, and while they are insensitive to inhibition by NBMPR, can be distinguished functionally based on their permeant selectivities (2). Human CNT1 and CNT2 both transport uridine and certain uridine analogs, but are otherwise selective for either pyrimidine (hCNT1) or

purine (hCNT2) nucleosides, except for modest transport of adenosine by hCNT1 (18). In contrast, hCNT3 transports both purine and pyrimidine nucleosides. All of the human nucleoside transporters described to date can transport adenosine to some extent, and may therefore be relevant to its signaling.

The aim of the present study was to identify potential factors that control adenosine homeostasis, and to determine their impact on ion transport in human airway epithelial cells. Under normal conditions, adenosine is present in the airway surface liquid where it mediates cilia beat frequency and mucus secretion (21). For these reasons, adenosine has received significant attention as an endogenous regulator of mucociliary clearance and as a potential therapeutic target for diseases, such as cystic fibrosis, characterized by abnormalities in this process. The results of our study show that equilibrative nucleoside transporters, together with adenosine kinase and 5'-nucleotidase play a major role in the control of adenosine effects in Calu-3 cells.

MATERIALS AND METHODS

A) Cell culture:

Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 5 μg/ml gentamycin sulfate, 6 μg/ml penicillin-G and 10 μg/ml streptomycin. Cells were maintained in T75 tissue-culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 5% CO_2 in air, and typically required 6-8 days to reach confluence. Confluent cell layers were passaged using saline solution containing 0.05% trypsin and 0.02% EDTA. Cells were seeded at a density of 5 x 10^5 cells/cm^2 onto Costar Transwell inserts (0.45-μM pore size, 0.33 cm^2 surface area) for adenosine transport experiments and onto Costar Snapwell inserts (0.45-μM pore size, 1 cm^2 surface area) for short-circuit current measurements. For the first six days, cells were grown submerged in culture medium that was changed every two to three days. Subsequently, air interface culturing was used, in which the medium was added only to the basolateral side of the inserts. Inserts were used for experiments 10-16 days after the establishment of an air interface. For patch clamp studies, 1 x 10^5 cells were seeded onto 15-mm coverslips (Fisherbrand, Pittsburgh, PA) 24 hours prior to experiments.

B) RT-PCR:

A portion (1.5 x 10^7) of cells harvested from T75 flasks were used for RNA purification using the Qiagen RNeasy kit (Qiagen), typically yielding 35 μg total RNA. First-strand cDNA was synthesized by reverse transcription of the RNA using Superscript II RNase H

Reverse Transcriptase (Invitrogen) and random hexamer primers (200 ng). Thereafter, PCR was performed using the following sets of primers (from 5' to 3') and annealing temperatures: A_1 (GenBank™ accession number L22214) forward nucleotides 603-623, reverse 847-826, at 55°C; A_{2A} (U40771) forward 546-569, reverse 995-972, at 55°C; A_{2B} (X68487) forward 447-466, reverse 958-938, at 55°C; A_3 (L22607) forward 353-376, reverse 793-770, at 55°C; hENT1 (U81375) forward 623-644, reverse 950-930, at 58°C; hENT2 (AF029358) forward 410-430, reverse 751-731, at 58°C; hCNT1 (U62968) forward 1-23, reverse 612-593, at 58°C; hCNT2 (AF036109) forward 1-22, reverse 540-521, at 58°C; hCNT3 (AF305210) forward 864-887, reverse 1319-1295, at 57°C. In addition to the primers designed to amplify sequences of interest, reactions with glyceraldehyde-3-phosphate dehydrogenase specific primers were run in all rounds of PCR reactions to serve as internal positive controls: GAPDH (accession number M33197) forward 212-235, reverse 806-786, at 60°C. Primers were obtained from Invitrogen (Carlsbad, CA). PCR was performed using the hot-start method, where one tenth of the reverse transcription reaction was combined with 1 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 U of Taq polymerase, and autoclaved distilled water to a final volume of 20 μl. After 5 min at 94°C, amplifications proceeded under the following conditions: 30 cycles (94°C, 45 s; annealing temperature, 30 s; 72°C, 90 s) with a final elongation period at 72°C for 7 min. PCR products were separated and visualized by electrophoresis on ethidium bromide-stained 1.5% agarose gels. The expected sizes (bp) of the PCR products were: 245 (A_1), 450 (A_{2A}), 512 (A_{2B}), 441 (A_3), 327 (hENT1), 341 (hENT2), 613 (hCNT1), 541 (hCNT2), 456 (hCNT3) and 595 (GAPDH). To confirm their identities, all RT-PCR

products were sequenced in one (adenosine receptors) or both (nucleoside transporters) directions by Taq dideoxyterminator cycle sequencing using an automated Model 373A DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing was done by the University of Alberta DNA Sequencing Core Facility.

C) Nucleoside transport:

Experiments were carried out at 20°C in HEPES-buffered Ringer's solution (HPBR) containing (in mM) 135 NaCl, 5.0 KCl, 3.33 NaH_2PO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 5.0 HEPES (pH = 7.4 at 20°C) or in Na^+-free HPBR containing (in mM) 140 N-methyl-D-glucamine (NMG), 5.0 KH_2PO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 5.0 HEPES (pH = 7.4 at 20°C). Confluent monolayers of Calu-3 cells, grown on permeable filters, were washed six times with HPBR or Na^+-free HPBR and then pre-incubated in the same solution (± 1 μM NBMPR) for 30 min. Uptake was initiated by adding 10 μM ^{14}C-labelled adenosine (0.5 μCi/ml, Amersham Pharmacia Biotech) in HPBR or Na^+-free HPBR (± 1 μM NBMPR) to either the apical or basolateral compartment. Incubation buffer also included 1 μM deoxycoformycin to inhibit ADA activity. Uptake was terminated by ten rapid washes of the cell culture inserts in an ice cold "stop" solution containing (in mM) 100 $MgCl_2$ and 10 Tris-HCl (pH = 7.4 at 0°C). The monolayers were dissolved in 0.2 ml 5% (w/v) SDS and counted for radioactivity using a Beckman LS 6000IC liquid scintillation counter (Irvine, CA). Non-mediated (passive) uptake was determined in the presence of 1 μM NBMPR and excess (5 mM) unlabeled uridine. The protein content of representative monolayers was measured using the Bio-Rad Protein

Standard Assay Procedure. The flux values are expressed in pmol/(mg protein · min) as means ± SEM.

D) Transepithelial measurements:

Standard techniques were used in Ussing chamber studies. The tissues were bathed on apical and basolateral sides with 10 ml of Krebs-Henseleit (KH) solution, which was warmed to 37°C and continually circulated with a gas lift using 95% O_2-5% CO_2. The composition, in mM, was 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4. Chemicals were added from concentrated stock solutions and both chambers were continuously and separately perfused to ensure proper oxygenation and stirring of the solutions. The transepithelial potential difference was clamped to zero using a DVC 1000 voltage/current amplifier (WPI, Sarasota, Fl) and the resulting short circuit current (I_{sc}) was recorded through Ag-AgCl electrodes and 3 M KCl agar bridges. The I_{sc} was allowed to stabilize for 30 min prior to the application of adenosine receptor agonists, and all experiments were performed in the presence of 10 μM apical amiloride. Positive currents were defined as anion secretion or movement from basolateral to apical side. The data were collected at 10 Hz and stored using a PowerLab 8SP series (ADInstruments, NSW 2154, Australia). Average ΔI_{sc} was calculated as the mean current in the first 300 sec after drug addition.

E) Patch-clamp recordings:

Pipette electrodes were made from thin-walled borosilicate glass (A-M Systems Inc., Everett, WA) using a two-stage vertical puller (Narishige, Japan). Electrode tips were fire

polished to a final resistance of 3-6 MΩ for whole-cell recordings and 8-10 MΩ for cell-attached recordings. Cells, cultured on 15-mm round coverslips, were rinsed three times in bath solution immediately before being mounted into an open bath chamber (Warner Instruments Inc., Hamden, CT), maintained at 37°C and fixed to the stage of an Olympus IMT-2 Inverted Research Microscope (Lake Success, NY). After the pipette had been immersed in bath solution, offset potentials were compensated before forming a GΩ seal. Currents were recorded, using an Axopatch 200A amplifier and Clampex 8.0 software, both from Axon Instruments (Foster City, CA). All currents were reported with reference to zero in the bath and data were analyzed by pClamp 8.0 (Axon Instruments) and Microcal Origin 6.0 (Northampton, MA) software.

Whole-cell recordings. The pipette solution contained (in mM): 5 NaCl, 135 KCl, 1.8 $MgCl_2$, 0.2 $CaCl_2$, 1 MgATP, 0.2 LiGTP, 10 glucose, 0.5 EGTA and 10 HEPES (pH 7.4). Bath solution contained (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.4). Once the whole-cell configuration was obtained the cell was clamped to –40 mV. The cell capacitance and the access resistance of the patch were determined directly by the compensation circuitry of the patch clamp amplifier, as well as by Clampex 8.0 software. The whole-cell capacitance, expressed as a mean ± S.D., was 49 ± 18 pF (n=105) and only seals with a series resistance of less than 20 MΩ were analyzed. Current recordings were obtained at 1 min intervals in response to voltage steps, each lasting 200 ms, from –80 mV to 80 mV in 20 mV increments. Traces were normalized to 1 pF, in order to remove variability due to differences in cell size, and the current-voltage relationship was obtained from the mean current during the central 140 ms of each recording.

Cell-attached recordings. Both the bath and pipette solution contained (in mM): 160 Tris Cl, 30 sucrose (pH 7.0). Once the seal was obtained, the pipette potential was clamped to –40 mV. Continuous recordings of channel activity, sampled at 5 kHz, were made. Voltage was briefly stepped from –80 to 80 mV in 20 mV increments to obtain a current-voltage relationship. All kinetic data were obtained at –80 mV, where CFTR Cl^- channel activity was most apparent. Recordings were filtered at 100 Hz, using an 8-Pole Bessel Filter; current-amplitude histograms were made and fit with Gaussian functions. The closed probability (P_C) was calculated as the proportion of the area under the curve that corresponded to the state in which all channels were closed. The open probability (P_O) was then calculated, for patches that contained one or more CFTR channels, as the total area under the curve minus the closed probability ($1-P_C$).

F) Chemicals:

Stock solutions were prepared in H_2O for adenosine (10 mM), erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 1 mM), deoxycoformycin (1 mM), 2-p-(2-carboxyethyl)phenethylamino-5'-(N-ethylcarboxamido)adenosine (CGS-21680, 1 mM), α,β-methylene-ADP (100 mM), 8-(p-sulfophenyl)theophylline (8-SPT, 10 mM), and amiloride (10 mM). Stock solutions of NBMPR were in methanol (3 mM), of 5'-(N-ethylcarboxamido)adenosine (NECA) were in 0.1 N HCl (10 mM), and of 5'-amino-5'-deoxyadenosine were in 1:1 (v:v) glacial acetic acid:water (20 mM). All the above drugs were obtained from Sigma (St. Louis, MO).

G) Data analysis:

Data are presented as means ± SEM, unless otherwise indicated; n refers to the number of experiments. The paired Student's t-test was used to compare the means of two groups. Statistically significant differences among the means of multiple groups were determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer posttest using Graphpad Instat 3.05 software (San Diego, CA). A value of $P<0.05$ was considered statistically significant.

RESULTS

A) Identification of adenosine receptors and transporters using RT-PCR:

The regulatory actions of adenosine are mediated via four subtypes of G-protein coupled receptors known as A_1, A_{2A}, A_{2B} and A_3. Using RT-PCR, we found that A_{2A} and A_{2B} mRNAs were present in Calu-3 cells at relatively high concentrations, with the A_{2B} signal being the strongest (Fig. 3.1A). The mRNAs for the A_1 and A_3 receptors were not detected. Figure 3.1B shows RT-PCR amplification of nucleoside transporter transcripts in Calu-3 cells. The cells expressed mRNA for the equilibrative nucleoside transporters, hENT1 and hENT2, and the concentrative nucleoside transporter hCNT3, whereas mRNA transcripts for hCNT1 and hCNT2 were not detected. All PCR products were sequenced and found to be identical to the corresponding GeneBank™ sequences. Control amplifications in which mRNA was used as a template for PCR, or in which no template was used, were negative for reactions involving adenosine receptors (A_{2A} and A_{2B}), and nucleoside transporters (hENT1, hENT2, and hCNT3). Control amplifications with GAPDH primers were always positive.

B) Polarized distribution of adenosine receptors:

We used short circuit current (I_{sc}) measurements as a functional assay to determine the distribution of adenosine receptors in apical and/or basolateral membranes. The experiments were performed using CGS-21680, a specific A_{2A} receptor agonist, and NECA, a non-specific adenosine receptor agonist. Representative recordings that demonstrated the effects of sequential addition of CGS-21680 (1 μM) and NECA (10

μM) on I_{sc} are shown in Figure 3.2. Baseline I_{sc} was 17 ± 4 μA/cm^2 (n=10), expressed as a mean ± SD. CGS-21680 consistently activated I_{sc} when added to the basolateral aspect of the monolayer regardless of the presence or absence of apical CGS-21680. In contrast, application of CGS-21680 to the apical membrane induced only a small I_{sc} increase (0.9 ± 0.3 μA/cm^2), which was not statistically significant (P>0.05, n=5). Furthermore, when CGS-21680 was added to the apical membrane in the presence of basolateral CGS-21680, there was never any effect on I_{sc} (n=5). Thus, A_{2A} receptors play a significant role in regulating anion secretion but only from the basolateral side.

The role of A_{2B} receptors was examined by applying NECA to each side of the monolayer in the presence of bilateral CGS-21680. Because RT-PCR experiments demonstrated the expression of only A_{2A} and A_{2B} receptor subtypes in Calu-3, this protocol allowed for the assessment of A_{2B} function despite the lack of specific agonists and antagonists. Under these conditions NECA stimulated an increase in I_{sc} when applied to either the apical or the basolateral surface (Fig. 3.2). However, the average ΔI_{sc} was greater when NECA was applied to the apical membrane first rather than the basolateral membrane. Furthermore, the effects of apical and basolateral NECA were not additive. Apical NECA stimulated the I_{sc} by 9.3 ± 1.5 μA/cm^2 (n=5), which could not be further increased by subsequent addition of basolateral NECA. Basolateral NECA stimulated the I_{sc} by 5.2 ± 0.7 μA/cm^2 (n=5), which was further increased by an additional 3.8 ± 0.9 μA/cm^2 (n=5) by apical NECA.

C) Polarized distribution of nucleoside transporters:

Uptake of ^{14}C-adenosine (10 μM) was measured as a function of time across both the apical and basolateral membranes. The uptake was linear for at least 3 min on both sides (apical < basolateral, R=0.989 and 0.975, respectively), therefore all subsequent initial rate measurements were carried out using 2-min incubations.

Adenosine uptake activities were further examined in detail to determine which transporter types were functional in each membrane (Fig. 3.3). The transport of adenosine was not significantly reduced by removal of extracellular Na^+ in either the apical or the basolateral membrane. However, addition of 1 μM NBMPR, a concentration sufficient to block all hENT1-mediated transport activity, substantially inhibited adenosine uptake across both membranes. Furthermore, when excess unlabelled uridine (5 mM), which saturates nucleoside transporters and effectively blocks transporter-mediated adenosine uptake, was added to the basolateral side, an NBMPR-insensitive Na^+-independent process was evident. Any remaining fluxes in the apical and basolateral membranes were attributed to passive (non-transporter mediated) processes. These experiments identified hENT1 activity as the dominant transport process in the apical membrane, while both hENT1 and hENT2 were functional in the basolateral membrane. When corrected for passive uptake, the ratio of hENT1 to hENT2 activity at the basolateral surface was 3:2.

D) The effect of exogenous and autocrine adenosine on whole-cell current:

Figure 3.4 shows typical recordings of the whole-cell current in Calu-3 cells. Under our experimental conditions the reversal potentials for K^+, Na^+ and Cl^- currents were -84.6 mV, 84.6 mV, and 0 mV, respectively. Measured baseline reversal potential, was -11 ±

0.5 mV (n=105), indicating that the majority of the whole-cell current was carried by Cl^- ions with some contribution by K^+ ions, while measured baseline whole-cell conductance was 127 ± 4 pS/pF (n=105). Addition of adenosine (100 μM) to the bath solution significantly activated whole-cell conductance (to 320 ± 36 pS/pF) and shifted the reversal potential to 1 ± 2 mV (n=8, Fig. 3.4B). This indicates that although both K^+ and Cl^- channels contribute to the baseline whole-cell current, adenosine predominantly activates chloride current.

The effect of autocrine adenosine on the whole-cell current was studied using NBMPR (1 μM) to inhibit uptake of extracellularly produced adenosine. We reasoned that this would increase extracellular adenosine, thereby leading to activation of adenosine receptors. Figure 3.4C shows that addition of NBMPR to the bath solution increased the whole-cell conductance (to 246 ± 28 pS/pF, n=6) in a manner that was similar to that observed with the addition of exogenous adenosine. In particular, NBMPR shifted the reversal potential to −1 ± 2 mV, consistent with Cl^- current activation. The requirement for adenosine receptors was confirmed by demonstrating that addition of NBMPR in the presence of the non-selective adenosine receptor antagonist, 8-SPT (1 μM), had no effect on the whole-cell conductance when compared to the baseline (107 ± 36 pS/pF, n = 4, paired Student *t*-test). Interestingly, 8-SPT alone inhibited basal whole-cell conductance (to 42 ± 8 pS/pF, n=4, $P<0.001$). This indicates that endogenous adenosine is formed extracellularly under baseline conditions.

E) Enzymes involved in adenosine produ[illegible]d metabolism control whole-cell current:

Figure 3.5A shows the effect of the adenosine kinase inhibitor, 5'-amino-5'-deoxyadenosine (20 μM, n=4) on the whole-cell current. The activated conductance (350 ± 29 pS/pF, n=4) was similar to that seen with the application of exogenous adenosine. This fact suggests that under baseline conditions adenosine kinase converts adenosine to AMP, thereby lowering its effective concentration in the vicinity of receptors. Like the effect of NBMPR, the effect of 5'-amino-5'-deoxyadenosine could be prevented by pre-treatment of the cells with 8-SPT (whole-cell conductance was 90 ± 17 pS/pF, n=3). Figure 3.5C shows the effect of the 5'-nucleotidase inhibitor, α,β-methylene-ADP (300 μM) on the whole-cell current. Inhibition of 5'-nucleotidase significantly reduced whole-cell conductance (to 29 ± 16 pS/pF) and shifted the reversal potential to –21 ± 2 mV (n=3), consistent with inhibition of chloride channel activity. This suggests that endogenous adenosine activates Cl^- channels under baseline conditions, which is in agreement with the results of experiments using 8-SPT.

In other studies, we used an inhibitor of ADA, EHNA (10 μM), to evaluate the role of this enzyme in adenosine homeostasis. There was no significant effect of EHNA on the whole-cell conductance over a period of 5 min, when added to either the pipette (180 ± 56 pS/pF, n=5) or bath (184 ± 73 pS/pF, n=3) solutions ($P>0.05$, compared with baseline). This suggests that adenosine deaminase does not play a significant role in adenosine metabolism in Calu-3 cells, under these conditions.

F) Inhibition of adenosine transporters or adenosine kinase activates CFTR chloride channels:

We used the single-channel patch clamp technique in the cell-attached mode, to identify ion channels activated by inhibition of adenosine transport or enzymes involved in adenosine metabolism. Figure 3.6 shows a representative recording of an ion channel under control conditions and after treatment with NBMPR. Since chloride was the only permeant ion present, and the channel conductance is 8 ± 3 pS (mean ± SD, n=13), this strongly suggests that inhibition of nucleoside transport by NBMPR activates CFTR Cl^- channels. NBMPR had no effect on the channel conductance (Fig. 3.6B), but significantly increased its open probability (Fig. 3.6C). Similarly, 5'-amino-5'-deoxyadenosine increased ion channel P_O from 0.04 ± 0.02 to 0.54 ± 0.10 (n=3, $P<0.05$), without changing its conductance.

DISCUSSION

Extracellular adenosine has been previously shown to modulate the function of both cation (20) and anion (4,12) channels in human airway epithelial cells. There are also reports suggesting that the presence of nucleoside transporters and enzymes involved in adenosine metabolism may regulate these effects by controlling the effective concentration of adenosine in the vicinity of its receptors (15,20). The results presented in this paper, and summarized in Figure 3.7, confirm and extend these observations by identifying enzymes that determine adenosine concentrations, and by characterizing adenosine receptors and nucleoside transporters involved in the regulation of ion channels in Calu-3 cells.

The regulatory actions of adenosine are mediated via four subtypes of G-protein coupled receptors, A_1, A_{2A}, A_{2B} and A_3 (17). Activation of each of these receptors has been linked to the regulation of ion transport in epithelial tissues (1,4,15). The results of our RT-PCR experiments show that Calu-3 cells express A_{2A} and A_{2B}, but not A_1 or A_3 receptors. The presence of A_{2B} receptors in Calu-3 cells has been reported previously (4). These receptors were localized predominantly to the apical side, with some presence at the basolateral membrane. Our data confirm that report and show, in addition, that A_{2A} receptors are present at the basolateral membrane.

Epithelial nucleoside transport has been most extensively studied in intestine, kidney, liver and choroid plexus (2). Enterocytes of the small intestine, for example, contain transcripts for all five of the CNT and ENT transporter isoforms and exhibit CNT1 and CNT2 functional activities in their apical membranes and ENT1 and/or ENT2 functional

activities in their basolateral membranes (reviewed in 26). Cultured T84 cells, a model of intestinal crypt cells, exhibit basolaterally-restricted ENT1 and ENT2 functional activities, while nucleoside uptake across the apical membrane has the characteristics of passive diffusion (14,23). Immunocytochemical analyses have demonstrated the presence of CNT1 protein in the apical membrane of rat small intestine, but not at the basolateral membrane, and a similar apical localization was identified for kidney proximal tubule (11). In rat liver parenchymal cells, CNT1 was abundant in bile canalicular membranes, but largely excluded from sinusoidal membranes (11) which, instead, are enriched in CNT2 immunoreactivity (7). Choroid plexus exhibits CNT3-type functional activity (24). Much less is known about nucleoside transport in other epithelia, although pharmacological and RT-PCR studies suggest the presence of CNT2 but not CNT1 in rat epididymal epithelium (13).

In the present study, we have used complementary molecular and functional approaches to investigate the nucleoside transport capabilities of Calu-3 cells. In the first series of experiments, RT-PCR was used in conjunction with isoform-specific oligonucleotide primers to test for the presence of hCNT1, hCNT2, hCNT3, hENT1 and hENT2 mRNA. In the second, transport studies were used to confirm the identity of the expressed nucleoside transporters, and to investigate their vectorial distribution (apical versus basolateral membrane). RT-PCR results show that Calu-3 cells express hENT1, hENT2 and hCNT3 mRNAs, but lack transcripts for hCNT1 and hCNT2. However, despite the presence of hCNT3 mRNA we failed to detect any Na^+-dependent adenosine transport activity in functional studies indicating a minor role, if any, for hCNT3 in adenosine transport in Calu-3 cells. hENT1 and hENT2 transport activities were detected

in basolateral membranes (hENT1 > hENT2), but only hENT1 activity was found in apical membranes. While hENT1 and hENT2 both transport adenosine and uridine and are broadly selective for other purine and pyrimidine nucleosides, the two transporters are not functionally equivalent. For example, hENT1 has generally higher apparent permeant affinities than hENT2, while hENT2 is capable of interacting with nucleobases and hENT1 is not (5,9,10). The two transporters may therefore fulfill complementary, but distinct physiological functions.

The results presented in this study show that application of NBMPR, a selective inhibitor of hENT1-mediated adenosine transport, had similar effects on whole-cell current as the application of exogenous adenosine. Furthermore, the effect of NBMPR was inhibitable by the adenosine receptor antagonist, 8-SPT, indicating that this effect was mediated through the activation of adenosine receptors. Therefore, adenosine transporters may regulate epithelial electrolyte secretion by controlling adenosine concentration in the vicinity of its receptors.

Enzymes involved in adenosine homeostasis exist in both intracellular and extracellular compartments. Adenosine is produced mainly by the action of 5'-nucleotidase, whereas its metabolism is controlled by adenosine kinase and ADA. The results presented in this study show that inhibition of 5'-nucleotidase by α,β-methylene-ADP significantly reduces whole-cell current. This is corroborated in experiments using the non-selective adenosine receptor antagonist 8-SPT, which also reduces basal whole-cell current. These observations suggest that adenosine is an endogenous regulator of Calu-3 chloride channel activity. Interestingly, similar manipulations to change endogenous adenosine levels had no effect on the whole-cell

current in A549 cells, another airway epithelial cell line (20). However, nucleoside transport activity in A549 cells is an order of magnitude greater then in Calu-3 cells. Therefore, nucleoside transporter activity may control the effectiveness of endogenous adenosine on epithelial ion transport.

Under physiological conditions, adenosine kinase is the main contributor to adenosine metabolism (6). Its K_m of 40 nM (19) is strategically positioned between the high affinity binding of adenosine to A_1 (3-30 nM), intermediate affinity binding to A_{2A} (20-200 nM) and low affinity binding to A_{2B} (5 μM) adenosine receptors (16). Inhibition of adenosine kinase would be expected to increase intracellular adenosine concentration, thereby leading to reversal of its normally inwardly directed concentration gradient, with release of adenosine and receptor activation. Our study supports this hypothesis since the inhibition of adenosine kinase by 5'-amino-5'-deoxyadenosine had similar effects on the whole-cell current as application of exogenous adenosine.

ADA is responsible for the hydrolytic deamination of adenosine to inosine. It has been shown recently that ADA-deficient mice exhibit a lung phenotype with features of asthma, including increased mucus secretion (3). These studies also showed that restoring ADA enzymatic activity to ADA-deficient mice attenuated mucus production, suggesting that, by regulating effective adenosine concentration, ADA plays an important role in the control of airway secretions. Interestingly, inhibition of ADA by EHNA had no significant effect on the whole-cell current. Therefore, ADA was probably not involved in the regulation of adenosine effects in Calu-3 cells under our experimental conditions. This may represent an attempt by the cells to conserve adenosine, by converting it to

AMP and trapping it intracellularly, rather than deaminating it to produce inosine, which is essentially a waste product.

Activation of CFTR Cl^- channels by adenosine has been shown in several studies (4,12). In particular, A_{2B} receptors in Calu-3 were shown to stimulate CFTR through a pathway involving activation of protein kinase A and phospholipase A_2 (4). Other studies suggested that A_{2B} receptors were coupled to CFTR by means of G_s proteins, adenylyl cyclase and protein kinase A (12). Our results are consistent with these studies but also show, for the first time, that inhibition of nucleoside transporters and enzymes involved in adenosine homeostasis, activates CFTR Cl^- channels. Thus, modulators of equilibrative nucleoside transporters or enzymes such as adenosine kinase or 5'-nucleotidase could play an important role in the regulation of CFTR-mediated anion secretion by adenosine receptors.

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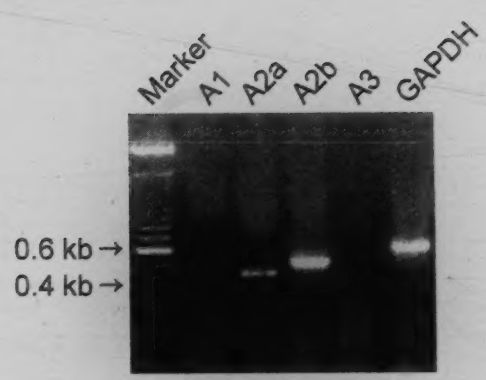
A.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A2a
A2b
A3
GAPDH
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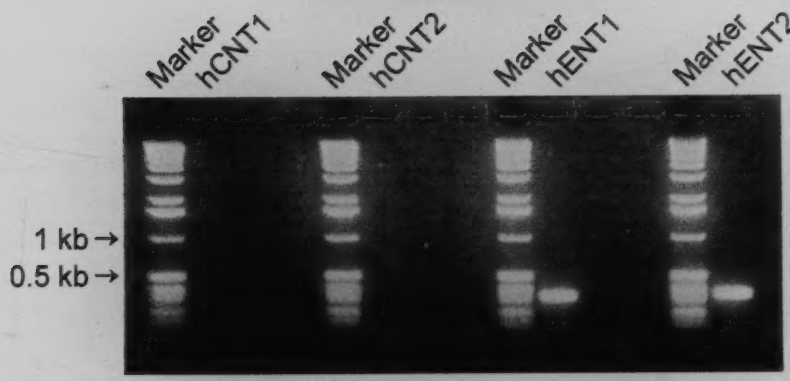
0.4 kb →

B.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Marker
hCNT2
Marker
hENT1
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hENT2
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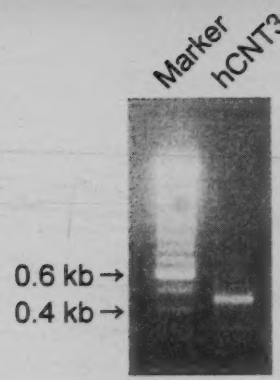
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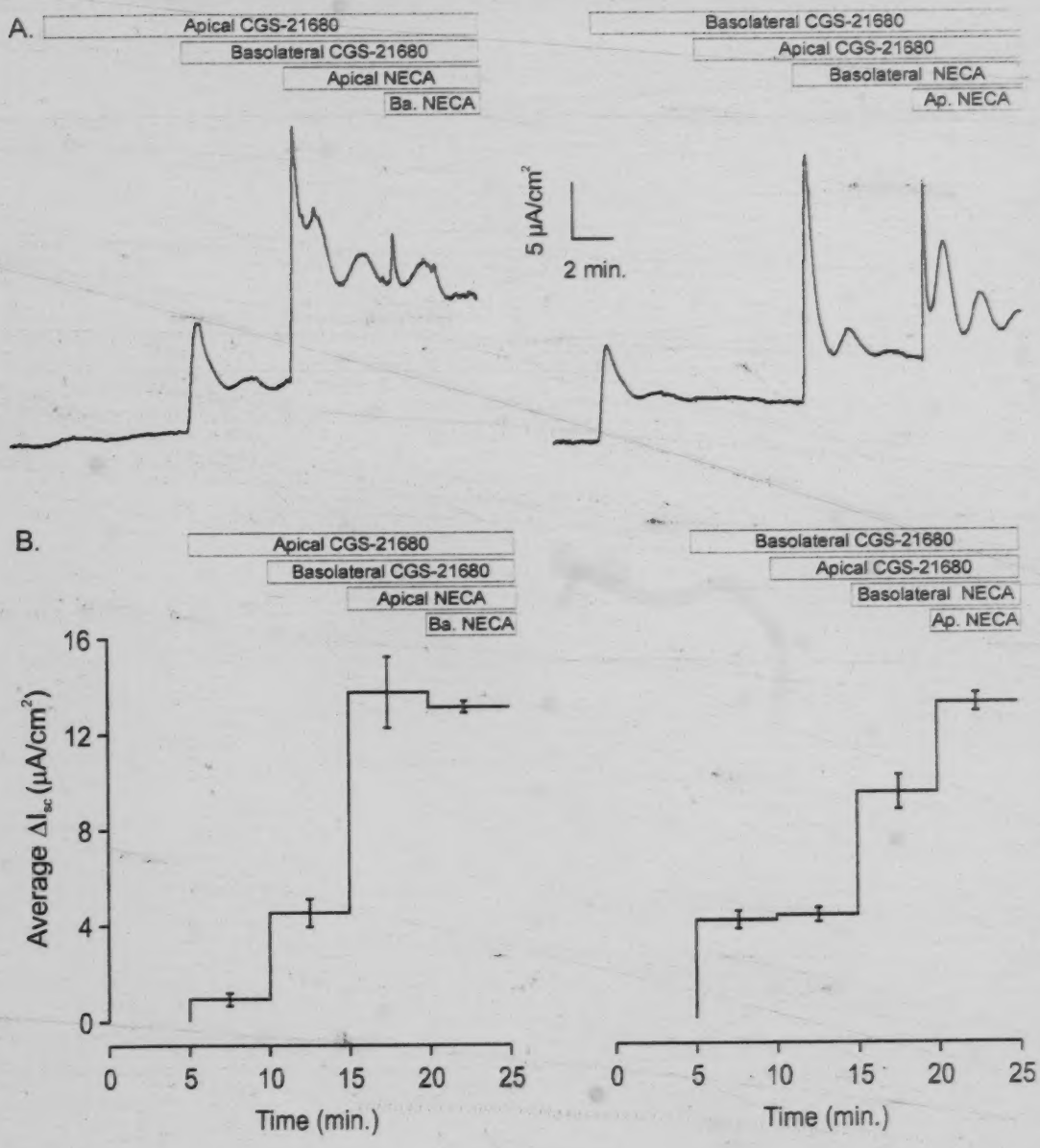
Figure 3.1


A.
Apical CGS-21680
Basolateral CGS-21680
Apical NECA
Ba. NECA
Basolateral CGS-21680
Apical CGS-21680
Basolateral NECA
Ap. NECA
5 μA/cm²
2 min.
B.
Apical CGS-21680
Basolateral CGS-21680
Apical NECA
Ba. NECA
Basolateral CGS-21680
Apical CGS-21680
Basolateral NECA
Ap. NECA
Average ΔIsc (μA/cm²)
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12
8
4
0
0
5
10
15
20
25
Time (min.)
Time (min.)

Figure 3.2

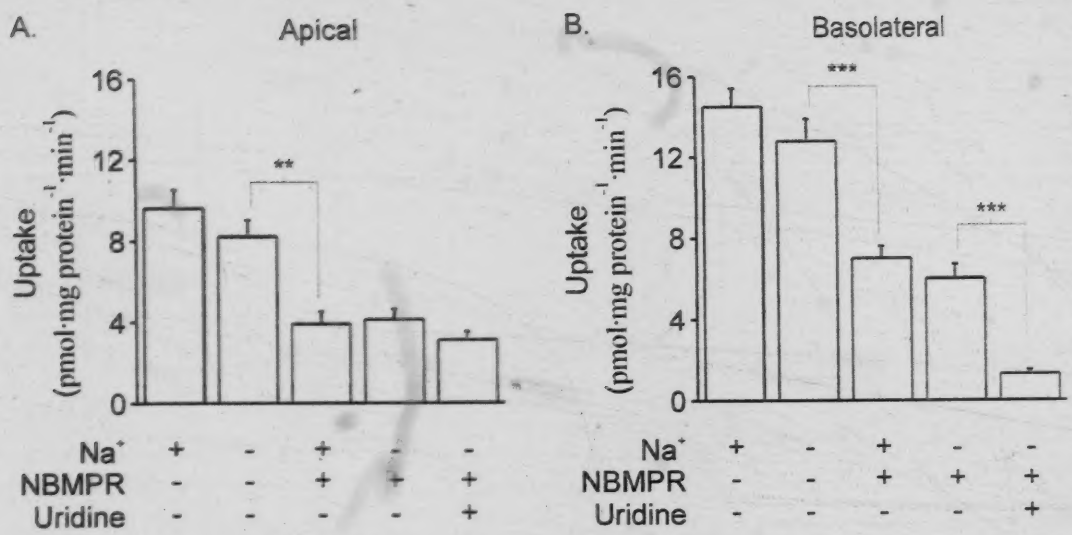
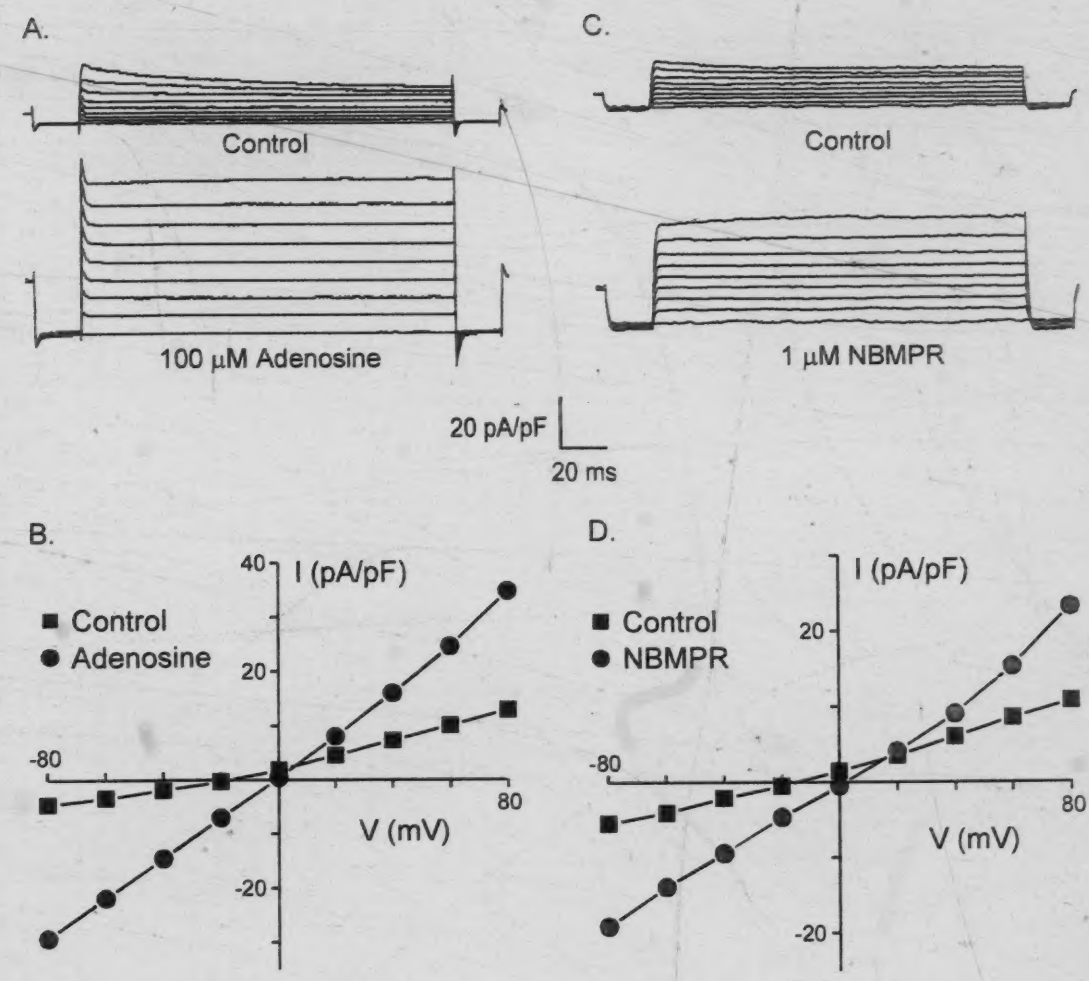

A.
Apical
B.
Basolateral
Uptake
(pmol·mg protein⁻¹·min⁻¹)
16
12
8
4
0
**
***
***
Na+ + - + - -
NBMPR - - + + +
Uridine - - - - +
Na+ + - + - -
NBMPR - - + + +
Uridine - - - - +

Figure 3.3


A.
Control
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C.
Control
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Control
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40
20
-80
80
V (mV)
-20
D.
I (pA/pF)
Control
NBMPR
20
-80
80
V (mV)
-20

Figure 3.4

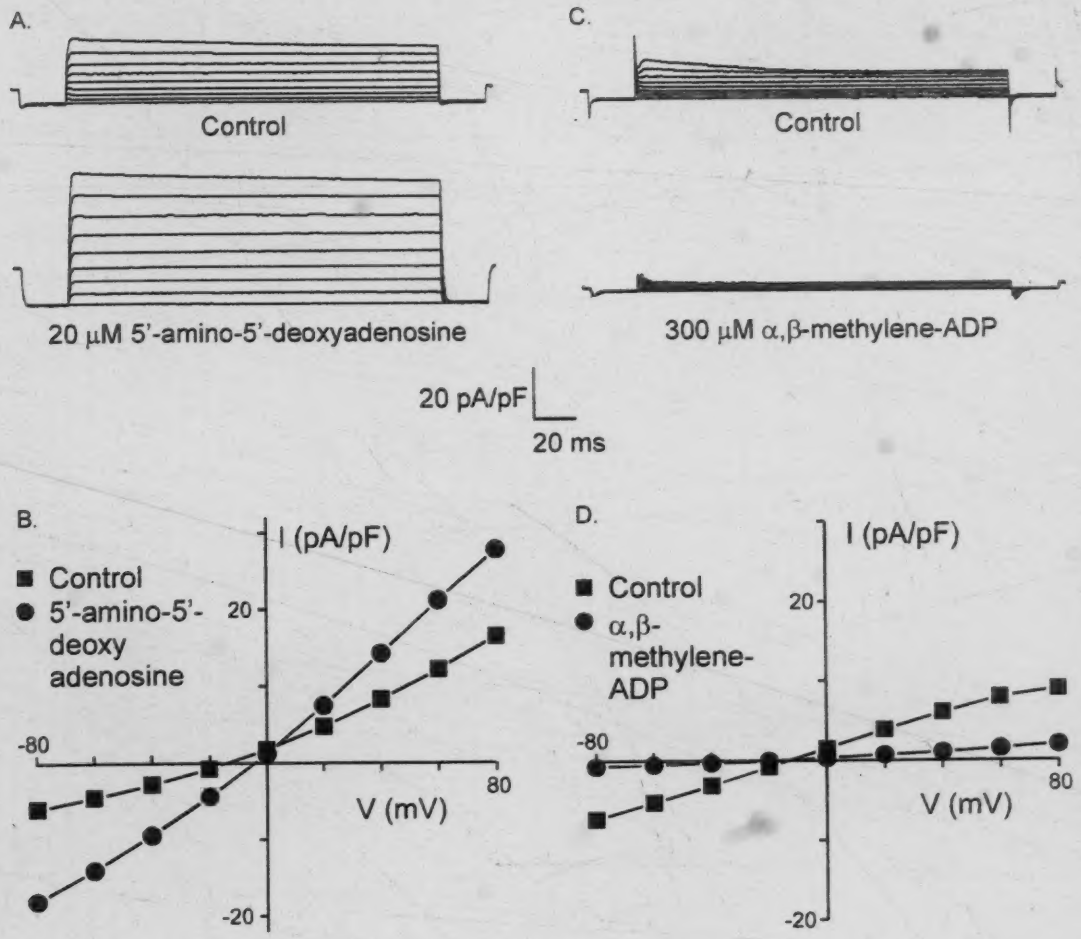
A.
Control
20 μM 5'-amino-5'-deoxyadenosine
C.
Control
300 μM α,β-methylene-ADP
20 pA/pF
20 ms
B.
I (pA/pF)
Control
5'-amino-5'-deoxy adenosine
20
-80
80
V (mV)
-20
D.
I (pA/pF)
Control
α,β-methylene-ADP
20
-80
80
V (mV)
-20

Figure 3.5

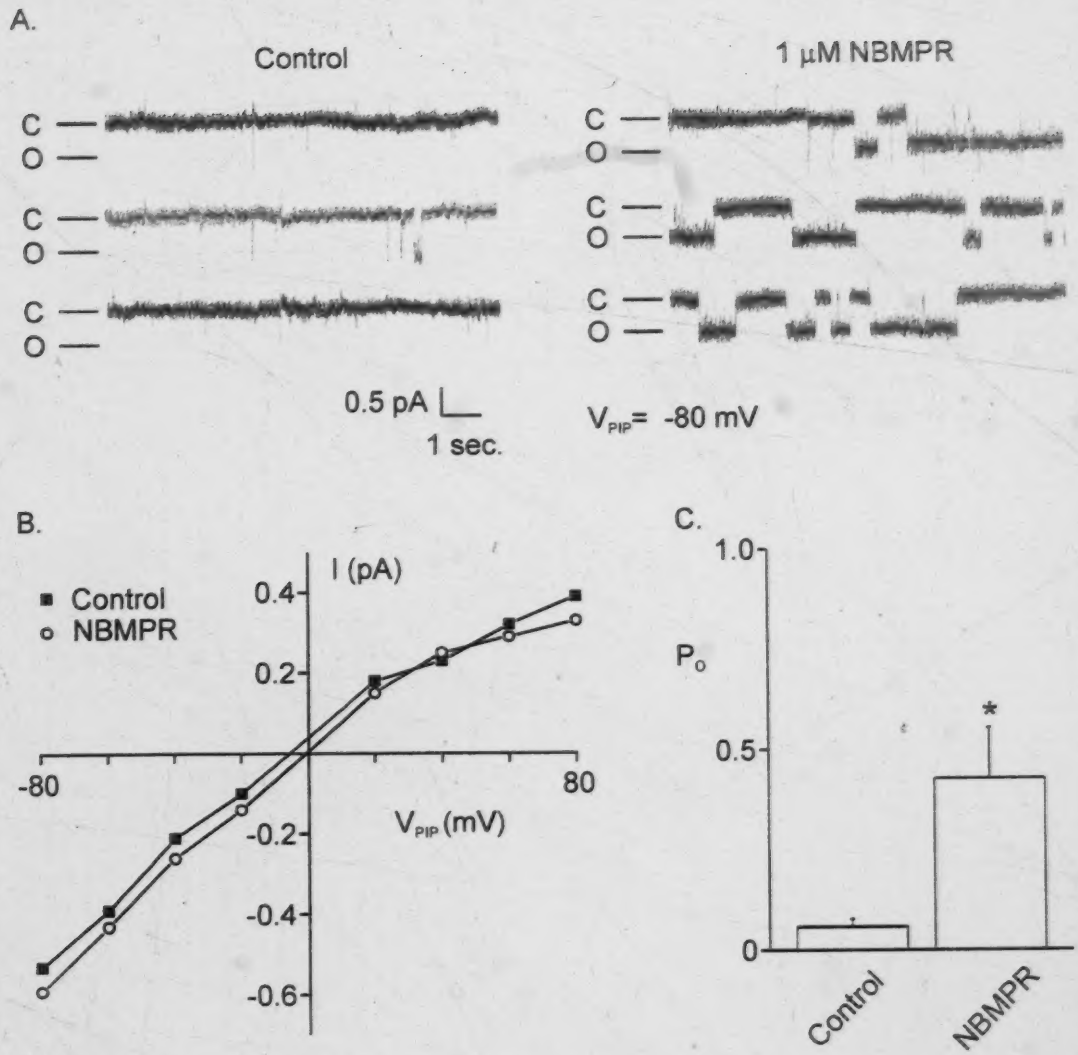
A.
Control
1 μM NBMPR
C
O
0.5 pA
1 sec.
V_{PIP}= -80 mV
B.
Control
NBMPR
I (pA)
0.4
0.2
-0.2
-0.4
-0.6
-80
80
V_{PIP} (mV)
C.
1.0
P_o
0.5
0
*
Control
NBMPR

Figure 3.6

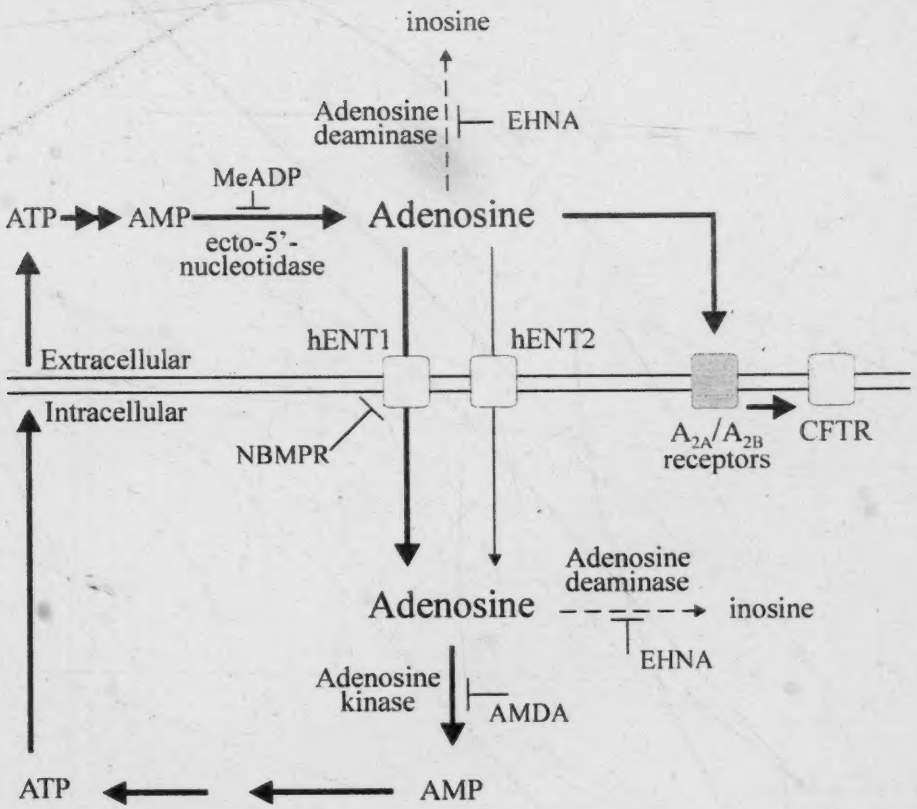
inosine
Adenosine deaminase
EHNA
MeADP
ATP
AMP
ecto-5'-nucleotidase
Adenosine
hENT1
hENT2
Extracellular
Intracellular
NBMPR
A_{2A}/A_{2B} receptors
CFTR
Adenosine deaminase
Adenosine
inosine
EHNA
Adenosine kinase
AMDA
ATP
AMP

Figure 3.7

FIGURE LEGENDS

Figure 3.1

RT-PCR analysis of adenosine receptors and transporters. Arrows indicate the positions of the marker fragments. ***A***: Calu-3 cells express transcripts for A_{2A} and A_{2B} but not A_1, and A_3 receptors. A representative positive control, using primers specific for GAPDH mRNA, is also included. ***B***: Transcripts for hENT1, hENT2, and hCNT3, but not hCNT1 and hCNT2 were detected. Equal amounts of cDNA were used for the PCR reactions.

Figure 3.2

Functional characterization of polarized adenosine receptor distribution. ***A***: Representative tracings showing the effects of 1 μM CGS-21680, a specific A_{2A} agonist, and 10 μM NECA, a nonspecific adenosine receptor agonist, on transepithelial short circuit current (I_{sc}). ***B***: A summary of experiments done with CGS-21680 in combination with NECA. All values are expressed as means ± SEM.

Figure 3.3

Functional characterization of polarized nucleoside transporter distribution. Initial rates of 10 μM ^{14}C-adenosine uptake [pmol/(mg protein·min)] across the apical (***A***) and basolateral (***B***) membranes were measured over a 2 min time course. In all experiments, nucleoside uptake was not significantly affected by the removal of Na^+, demonstrating the lack of concentrative Na^+/nucleoside co-transport activity. In contrast, adenosine

uptake across both apical and basolateral membranes was sensitive to inhibition by 1 μM NBMPR. In addition, NBMPR-insensitive transporter activity was detected on the basolateral membrane that could be blocked by saturating with excess unlabeled uridine (5 mM). The remaining nucleoside uptake was via passive (non-transporter mediated) mechanisms. Data are presented as means ± SEM of 20-62 inserts, *P<0.05, **P<0.01, ***P<0.001.

Figure 3.4

The effect of exogenous and autocrine adenosine on whole-cell current. ***A***: Representative traces showing the activation of whole-cell current by 100 μM adenosine (n=8), and the corresponding current-voltage plot (***B***). ***C***: Representative traces showing the activation of whole cell current by 1 μM NBMPR (n=6), and the corresponding current-voltage plot (***D***).

Figure 3.5

Enzymes involved in adenosine production and metabolism control whole-cell current. ***A***: representative traces showing the activation of whole-cell current by 20 μM 5'-amino-5'-deoxyadenosine (n=4), and the corresponding current-voltage plot (***B***). ***C***: Representative traces showing the inhibition of whole cell current by 300 μM α,β-methylene-ADP (n=3), and the corresponding current-voltage plot (***D***).

Figure 3.6

Nucleoside transport inhibition leads to activation of CFTR Cl^- channels. ***A***: Representative recordings of an ion channel under control conditions and after stimulation with 1 μM NBMPR. The recordings were obtained in the cell-attached patch clamp configuration, when pipette potential was clamped to -80 mV; the channel closed and open states are designated by C and O, respectively. ***B***: The current-voltage plot derived from the CFTR channel recording at various voltages, before and after NBMPR addition, showing that this drug does not influence channel conductance. ***C***: The graph shows the increase in P_O after NBMPR addition (n=4). *$P<0.05$.

Figure 3.7

Schematic diagram showing coupling of adenosine homeostasis to adenosine signaling in Calu-3 cells. Intracellular adenosine is rapidly metabolized to AMP by adenosine kinase such that its concentration is normally very low. Intracellular AMP can be converted to ATP, which can exit cells through mechanisms that are not completely understood. Extracellular ATP is converted to AMP by phosphatases, which is then metabolized to adenosine by ecto-5'-nucleotidase. This adenosine is available for either cellular uptak or receptor stimulation. Uptake occurs through hENT1, and to a lesser extent hENT2. Adenosine that is not taken up can stimulate A_{2A} or A_{2B} receptors leading to CFTR activation. The most important pathways are indicated with thick arrows, those of lesser importance are indicated with thin arrows, and those that appear to be unimportant in Calu-3 cells are indicated with dashed arrows. Abbreviations: 5'-amino-5'-deoxyadenosine (AMDA), α,β-methylene-ADP (MeADP).

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CHAPTER 4

The role of the basolateral ORCC in human airway epithelial anion secretion

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INTRODUCTION

Anion secretion by airway epithelial cells acts as a driving force for hydration of the airway surface liquid and is therefore a major determinant of mucociliary clearance. The transport pathways comprising the Cl^- and HCO_3^- secretory mechanism have been extensively studied (5,21,28). Cl^- enters the cell across the basolateral membrane via a Na^+-K^+-$2Cl^-$ (NKCC) cotransporter, which represents a secondary active transport process driven by electrochemical gradients established by the basolateral Na^+/K^+-ATPase. K^+ ions are recycled via basolateral K^+ channels and serve to adjust membrane potential, while vectorial secretion of Cl^- is achieved by its exit through apically localized anion channels. HCO_3^- ions are thought to enter the cell via basolaterally localized Na^+-HCO_3^- cotransporters, and to exit the cell through apical anion channels, which serve as a final common pathway for both Cl^- and HCO_3^- transport. It is well established that the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), the channel mutated in individuals with Cystic Fibrosis (CF), is the primary apical Cl^- conductance. However, it has only recently been shown that this channel is also responsible for conducting HCO_3^- across the apical membrane of airway epithelia. Illek *et al.* (13) have shown that basolaterally permeabilized Calu-3 cells, a human model of serous submucosal airway epithelium, mounted in a HCO_3^- gradient will conduct this anion via CFTR. Similarly, Paradiso *et al.* (18) used pH sensitive dyes to show that human nasal epithelia will acidify/alkalinize across their apical membranes via CFTR. This is further supported by the finding that the apical membranes of these cells do not display anion exchange activity, which could account for apical HCO_3^- translocation (17,18).

The finding that CFTR conducts Cl^- and HCO_3^- poses new questions, in particular it is unclear how cells control which anion is secreted at any given time. It has been suggested that opening of basolateral K^+ channels, which causes cell hyperpolarization, elicits a switch from HCO_3^- to Cl^- secretion (5,25). Hyperpolarization inhibits basolateral Na^+-HCO_3^- cotransporters, and therefore HCO_3^- secretion, while promoting Cl^- secretion by increasing the driving force for apical exit of this anion. Thus, opening of basolateral K^+ channels could explain a switch from HCO_3^- to Cl^- secretion. However, the mechanism by which switching in the opposite direction occurs, from Cl^- to HCO_3^- secretion, remains unknown. Presumably, reversing the logic and closing K^+ channels would not suffice in switching secretion from Cl^- to HCO_3^- because this would depolarize cells and therefore inhibit secretion alltogether. Furthermore, the mechanism by which HCO_3^- secretion can dominate Cl^- secretion at any given moment is unknown, particularly considering that Cl^- is both more abundant than HCO_3^- and 4 to 10 times more permeable through CFTR channels (8,16).

Prior to the cloning of CFTR, the outwardly rectifying Cl^- channel (ORCC) was suspected of being the primary defect in CF when it was shown that this channel could be activated by cAMP-dependent mechanisms in normal, but not in CF airway epithelia (6,27). After the cloning of CFTR, it was shown that CFTR regulates ORCC such that, in CF ORCC is unresponsive to cAMP-dependent stimuli (7). Schwiebert *et al.* (20) proposed a mechanism for this regulation, suggesting that CFTR mediates the autocrine release of ATP, which stimulates purinergic receptors and leads to activation of the ORCC. More recently, Xia *et al.* (30) used single-channel patch clamp studies of Calu-3 cells to demonstrate that ORCCs are rarely found in the apical membranes of confluent

layers, but are abundant in non-polarized dispersed cells. Thus, suggesting that these channels could be localized to the basolateral membrane.

Despite the ongoing interest in the ORCC, few anion transport models have proposed a clear physiological role for this channel in airway epithelial cells. Willumsen *et al.* (28) suggested that these channels contribute to Cl^- secretion by providing an additional pathway for Cl^- entry across the basolateral membrane, thereby supplementing the electrically silent cotransport system. However, this does not explain how vectorial Cl^- transport could be achieved, under short-circuit conditions, if both the apical and basolateral membrane transport processes are passive. Other studies suggest that the presence of a basolateral ORCC may facilitate cAMP-dependent Cl^- absorption across airway epithelium, under open-circuit conditions (26).

In previous studies we have shown that adenosine A_2 receptor stimulation leads to the activation of CFTR Cl^- channels in Calu-3 cells (23). During the course of those experiments we observed that anion channels, other than CFTR, were frequently activated by adenosine receptor stimulation. Therefore, the purpose of the present study was to characterize the role of these channels in transepithelial anion secretion in Calu-3 cells, and in primary cultures of Normal Human Bronchial Epithelium (NHBE). The results of our study show that adenosine not only activates CFTR, but also basolateral ORCCs, via a process that is cAMP-dependent and may involve A Kinase Anchoring Proteins (AKAPs). The fact that inhibition of these channels increases Cl^- secretion indicates that at least some of the Cl^- ions that enter the cell via NKCC cotransport are recycled across the basolateral membrane. Furthermore, we propose a physiological role for basolateral Cl^- channels as a molecular switch that controls the type of anion secreted.

Opening of basolateral Cl^- channels allows for Cl^- recycling across the basolateral membrane and therefore preferential HCO_3^- secretion, whereas closing of these channels favors Cl^- secretion.

MATERIALS AND METHODS

A) Cell Culture:

Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in DMEM supplemented with 10% FBS, 5 μg/ml gentamycin sulfate, 6 μg/ml penicillin-G and 10 μg/ml streptomycin. The cells were maintained in T75 tissue-culture flasks (Costar, Cambridge, MA), and typically required 6-8 days to reach ~85% confluence. At this time the cells were passaged using saline solution containing 0.05% trypsin and 0.02% EDTA. Cells were seeded at a density of 3.5×10^5 cells/cm^2 onto Costar Snapwell inserts (0.45-μm pore size, 1 cm^2 surface area) for short-circuit current (I_{sc}) measurements, and maintained in medium which differed only in that it contained 20% FBS. For the first six days, cells were grown submerged in culture medium. Subsequently, air interface culturing was used, in which the medium was added only to the basolateral side of the inserts. Inserts were used for experiments 10-16 days after the establishment of an air interface. For patch clamp studies, 1×10^5 cells were seeded onto 15-mm coverslips (Fisherbrand, Pittsburgh, PA) 24 hours prior to experiments. All cells used for experiments were at passage 24-27.

Normal Human Bronchial Epithelial (NHBE) cells were obtained from BioWhittaker (San Diego, CA) as frozen passage 1 stocks, containing ≥500,000 cells, and were cultured as described previously (4). The cells were thawed according to the instructions provided and seeded into T75 tissue culture flasks. They were grown in bronchial epithelial growth media (BEGM, BioWhittaker) supplemented with bovine pituitary extract (52 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth

factor (0.5 ng/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), triiodothyronine (6.5 ng/ml), gentamicin (50 μg/ml), amphotericin-B (50 ng/ml), penicillin G (6.2 μg/ml), streptomycin (10 μg/ml) and retinoic acid (330 nM). Retinoic acid (all-*trans*) was obtained from Sigma (St. Louis, MO), prepared in aliquots that were stored at -70°C, and was added to aliquots of media just prior to use. Care was taken to shield cells and media from light, particularly after supplementing with retinoic acid. When the cells reached 80% confluence they were passaged with trypsin/EDTA (BioWhittaker). A portion of the harvested cells was seeded into a new T75 flask at 3,500 cells/cm^2 at passage 2, while the rest were frozen at ≥500,000 cells/cryovial in 10% DMSO, for use later. Once 80% confluent the cells were passaged again, but now onto Costar Snapwell inserts (0.45-μM pore size, 1 cm^2 surface area) at 1 x 10^5 cells/insert, at passage 3. Cells plated onto inserts were maintained in media containing a 1:1 (v/v) mixture of BEGM:DMEM supplemented as above except that amphotericin-B and triiodothyronine were excluded, while retinoic acid was used at a reduced concentration (50 nM). The cells were grown submerged for the first 7 days, followed by air interface culturing for an additional 14 to 20 days. All NHBE cells used for experiments were at passage 3.

All cultures of both Calu-3 and NHBE cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Media was changed the day after seeding and subsequently every 2-3 days, until the desired confluence was reached.

B) Cell-Attached Patch Clamp:

Cells, cultured on 15-mm round coverslips, were rinsed three times in bath solution,

containing (in mM): 160 Tris Cl, 30 sucrose (pH 7.0). They were then mounted into an open bath chamber (Warner Instruments Inc., Hamden, CT), maintained at 37°C and fixed to the stage of an Olympus IMT-2 Inverted Research Microscope (Lake Success, NY). Pipette electrodes were made from standard borosilicate glass (Sutter Instrument Co., Novato, CA) using a two-stage vertical puller (Narishige, Japan), fire polished to a final resistance of 8-20 MΩ, and back-filled with bath solution. After pipette immersion in bath solution, offset potentials were compensated and a GΩ seal was formed. Currents were recorded, using an Axopatch 200A amplifier and Clampex 8.0 software, both from Axon Instruments (Foster City, CA). Continuous recordings of channel activity, sampled at 5 kHz, were made when voltages were clamped from -80 mV to 80 mV in 20 mV increments. All currents were reported with reference to zero in the bath and data were analyzed by pClamp 8.0 (Axon Instruments) and Microcal Origin 6.0 (Northampton, MA) software. Recordings were filtered at 100 Hz, using an 8-pole Bessel filter; current-amplitude histograms were made and fit with Gaussian functions. The closed probability (P_C) was calculated as the proportion of the area under the curve that corresponded to the state in which all channels were closed. The open probability (P_O) was then calculated, for patches that contained one or more non-CFTR channels, as the total area under the curve minus the closed probability ($1-P_C$). Voltage is expressed as the negative pipette potential ($-V_{PIP}$).

C) Transepithelial Measurements:

Standard techniques were used in Ussing chamber studies. Cells grown on inserts were bathed on apical and basolateral sides with 10 ml of Krebs-Henseleit Solution (KHS), or

a modified version there of (Table 4.1). Solutions were warmed to 37°C and continually circulated with a gas lift using either 95% O_2-5% CO_2 if the solution was HCO_3^- buffered, or 100% O_2 if the solution was HEPES buffered. Chemicals were added from concentrated stock solutions and both chambers were continuously and separately perfused to ensure proper oxygenation and stirring. The transepithelial potential difference was clamped using a DVC 1000 voltage/current amplifier (WPI, Sarasota, Fl) and the resulting current was recorded through Ag-AgCl electrodes and 3 M KCl agar bridges. In most cases the voltage was clamped to zero and the resultant I_{sc} was sampled at 10 Hz using a PowerLab 8SP series data acquisition converter and Chart software, both from ADInstruments (NSW 2154, Australia). Brief (1 s) pulses to 0.5 mV were applied every 90 s, in order to calculate resistances. All values are expressed as an average ΔI_{sc}, which was calculated as the mean change in current in the first 300 sec after drug addition, unless otherwise noted. During all experiments, the I_{sc} was allowed to stabilize for 20 min prior to treatments, and all experiments were performed in the presence of 10 μM apical amiloride.

When clamping at variable potentials was necessary, experiments were performed in the same way as described, with a few exceptions. The data were acquired at 100 Hz using the PowerLab 8SP series converter in conjunction with Scope software (ADInstruments); cells were clamped from -80 to 80 mV in 20 mV increments with each step lasting 1.5 s, and with brief (0.5 s) periods between each step at 0 mV. The DIDS-sensitive current was obtained by subtracting post-DIDS traces, from pre-DIDS traces. Positive currents were defined as anion secretion or movement from the basolateral to the apical side, and current-voltage relationships were obtained from the peak currents.

D) Radioisotopic Flux:

$^{36}Cl^-$ flux assays were carried out on Calu-3 inserts being used for simultaneous I_{sc} experiments in KHS. Inserts were short-circuited for 20 min. prior to the addition of the radioisotope. At time zero (T_0), samples for determination of background radioactivity were taken, followed by the addition of 3 μCi of $^{36}Cl^-$ (Amersham Pharmacia Biotech, UK) to the basolateral compartment, and another 20 min. allowed for the establishment of equilibrium. At this time (T_{20}) 0.5 ml samples were taken from the apical side and replaced with fresh KHS; this was repeated at every 10 min. interval thereafter. Three samples were taken (T_{20} - T_{40}) before the addition of bilateral NECA (10 μM), this was followed by two more samples taken (T_{50} and T_{60}) before the addition of basolateral DIDS (50 μM), and a further two more samples taken (T_{70} and T_{80}) after DIDS treatment. In addition, two samples were taken from the basolateral side, immediately before treatment with NECA, in order to calculate the specific activity. Samples were counted for radioactivity using a model 1219 Rackbeta (LKB, Finland) liquid scintillation counter. The unidirectional flux (J^{Cl}_{BA}) was calculated according to standard equations (19). $^{36}Cl^-$ fluxes in the apical to basolateral (J^{Cl}_{AB}) direction were measured in exactly the same fashion, except that the radioisotope was added to the apical bathing solution. Net $^{36}Cl^-$ flux (J^{Cl}_{net}) was calculated as $J^{Cl}_{net} = J^{Cl}_{BA} - J^{Cl}_{AB}$.

I_{sc} data represent the secretion of Cl^- and HCO_3^-, assuming that Na^+ absorption is negligible in the presence of amiloride (apical, 10 μM), and K^+ secretion is insignificant in Calu-3 cells (5). Therefore, net HCO_3^- flux ($J^{HCO_3}_{net}$) can be calculated as $J^{HCO_3}_{net} = I_{sc}$ -

J^{Cl}_{net}, where I_{sc} is the average short-circuit current expressed in flux units ($\mu Eq/cm^2 \cdot h$) over the 10 min period during which flux was assayed.

E) Chemicals:

Stock solutions were prepared in H_2O for 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 5 mM), 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS, 100 mM), 2-p-(2-carboxyethyl)phenethylamino-5'-(N-ethylcarboxamido) adenosine (CGS-21680, 1 mM), carbachol (100 mM) and amiloride (10 mM). Stock solutions of 9-anthracenecarboxylic acid (9-AC) were in 0.1 N NaOH (10 mM), 5'-(N-ethylcarboxamido)adenosine (NECA) in 0.1 N HCl (10 mM), furosemide in 0.1 N NaOH (100 mM), forskolin in 95% ethanol (10 mM), N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H-89) in 50% ethanol (5 mM), ionomycin in 95% ethanol (10 mM), nystatin in DMSO (90 mg/ml), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM) in DMSO (30 mM), and ouabain in KHS (10 mM), or a modified version there of (Table 4.1). DIDS and DNDS were purchased from Molecular Probes Inc. (Eugene, OR), forskolin from Calbiochem (San Diego, CA), and all other chemicals were from Sigma. InCELLect® stearated-Ht31 (St-Ht31) peptide and its analog St-Ht31P, a modified version of St-Ht31 in which two isoleucine residues are replaced with prolines, was obtained as a 10 mM stock in 50 mM Tris•HCl (pH = 7.5) from Promega (Madison, WI).

F) Data Analysis:

Data are presented as means ± SEM, unless otherwise indicated; n refers to the number of

experiments. The paired Student's *t*-test was used to compare the means of two groups. Statistically significant differences among the means of multiple groups were determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer posttest using Graphpad Instat 3.05 software (San Diego, CA). A value of $P<0.05$ was considered statistically significant.

RESULTS

A) Adenosine receptor stimulation activates ORCC:

Figure 4.1A shows representative traces of cell-attached patch clamp current recordings obtained before and after adenosine receptor agonist treatment (NECA, 10 μM). In cell-attached patches the channels were usually closed, but could be activated by NECA in 40% of patches (n=10). Figure 4.1B shows the current-voltage relationship obtained at voltages ranging from -80 to 80 mV. Activity was greatest at depolarizing potentials where the open probability was $P_O = 0.3 \pm 0.1$ and conductances were between 115 and 140 pS (n=4). These channels have been characterized previously in Calu-3 cells (9,30) and are known to be DIDS-sensitive (11), but the finding that they are activated by adenosine receptor stimulation is novel.

B) Basolaterally localized anion channels:

Further characterization of ORCC and their regulation by adenosine receptors was performed in transepithelial I_{sc} studies. In total, we evaluated 134 inserts with Calu-3 cells and 65 with NHBE cells. The basal I_{sc} and R_T in Calu-3 cells in normal Krebs-Henseleit solution (KHS) was 23.1 ± 1.1 μA/cm^2 and 210 ± 15 Ωcm^2 (n=59), respectively. Similarly, the basal I_{sc} and R_T in NHBE cells was 10.2 ± 0.8 μA/cm^2 and 811 ± 58 Ωcm^2 (n=28), respectively. In all experiments the cells were pre-treated with apical amiloride (10 μM), which had no effect on Calu-3 cells, but reduced baseline I_{sc} in NHBE cells to 2.9 ± 0.2 μA/cm^2 (n=28).

Adenosine receptor stimulation with bilateral NECA (10 μM) induced a biphasic I_{sc} response, with an initial peak followed by a plateau (Fig. 4.2). Overall, NECA increased I_{sc} by 40.5 ± 2.7 μA/cm^2 (n=10) in Calu-3 cells and by 20.9 ± 0.9 μA/cm^2 (n=3) in NHBE cells. The NECA response was abolished in the presence of a CFTR Cl^- channel blocker, DPC (1 mM, apical), in both cell types (n=4, P<0.005). Apical DIDS (50 μM) had no effect on either baseline or NECA-stimulated I_{sc}. However, basolateral DIDS caused a small but significant increase in baseline I_{sc} in Calu-3 cells (1.4 ± 0.1 μA/cm^2, n=3, P<0.05, Fig. 4.2B) and a much larger increase in cells pre-treated with NECA (15.6 ± 2.0 μA/cm^2, n=8, Fig. 4.2A). Similar results were obtained in NHBE cells, where DIDS only had an effect when applied basolaterally after NECA pre-treatment (4.2 ± 0.4 μA/cm^2, n=3). Interestingly, in the presence of basolateral DIDS, the NECA response was potentiated to 57.5 ± 2 μA/cm^2 (n=3, P<0.01, Fig. 4.2B) and 30.1 ± 2.9 μA/cm^2 (n=4, P<0.05) in Calu-3 and NHBE cells, respectively, bearing in mind that effects were quantified as the average I_{sc} in the first 5 minutes of the drug response. This suggests that basolateral DIDS-sensitive anion channels are stimulated through adenosine signaling in both cell types. However, it is difficult to gauge the magnitude of basolateral anion channel inhibition on I_{sc}, because DIDS is also known to block electrogenic Na^+-HCO_3^- cotransporters, and electroneutral Cl^-/HCO_3^- exchangers.

In an attempt to isolate the effect of basolateral anion channel inhibition, we used the alternative anion channel blockers - 9-AC and DNDS. In Calu-3 cells pre-treated with NECA, the application of basolateral 9-AC (1 mM) stimulated I_{sc} by 18.0 ± 0.9 μA/cm^2 (n=3, Fig. 4.2C). This effect was similar to that produced by DIDS (P > 0.05). However, other studies indicate that this high concentration of 9-AC is below the IC_{50} for ORCC

inhibition, and thus likely does not represent complete channel blockage (27). The application of basolateral DNDS (3 mM), in the presence of NECA, inhibited I_{sc} by 12.8 ± 1.6 μA/cm^2 (n=3, Fig. 4.2D), indicating that this drug does not block basolateral anion channels. Because both DNDS and DIDS inhibit Na^+-HCO_3^- cotransporters (and Cl^-/HCO_3^- exchangers), but only one appears to inhibit basolateral anion channels, the sequential application of these drugs allows us to isolate the contributions of each of these factors to the I_{sc}. In other words, when DIDS is applied after DNDS, the I_{sc} response reflects the inhibition of basolateral anion channels in isolation. Accordingly, the observed increase in I_{sc} induced by DIDS, in the presence of DNDS, is nearly doubled to 29.7 ± 1.1 μA/cm^2 (n=3, P<0.005, Fig. 4.2D), when compared to the application of DIDS alone.

C) The basolateral membrane contains ORCC:

Apical membrane permeabilization experiments were performed in order to further characterize the basolateral anion channels. These experiments were done in the presence of basolateral ouabain (1 mM), to inhibit the electrogenic Na^+/K^+-ATPase. Basolateral Na^+-K^+-$2Cl^-$ and Na^+-HCO_3^- cotransporters were also inhibited, by furosemide (1 mM) and HCO_3^- removal, respectively. An apical (HCO_3^- Free KHS) to basolateral (HCO_3^- Free, Low Cl^- solution) Cl^- gradient (129.1: 17.1 mM) was established prior to permeabilization (Table 4.1). Under these conditions, apical nystatin (90 μg/ml) increased I_{sc}, in Calu-3 cells, by -102 ± 13 μA/cm^2 (n=4, Fig. 4.3A) and -163 ± 20 μA/cm^2 (n=3, Fig. 4.3B) in the absence and presence of NECA, respectively (P<0.05). Furthermore, DIDS inhibited I_{sc} by 30.9 ± 1.0 μA/cm^2 (n=4, Fig. 4.3A) and

54.6 ± 10.9 μA/cm^2 (n=3, Fig. 4.3B) in the absence and presence of NECA, respectively (P<0.05). Similar results were obtained in NHBE cells where nystatin stimulated I_{sc} was larger in NECA pre-treated cells (-62.1 ± 2.6 μA/cm^2, n=4), than in non-treated cells (-45.5 ± 0.2 μA/cm^2, n=4, P<0.01). DIDS reduced I_{sc} by 14.1 ± 0.7 μA/cm^2 and 7.6 ± 0.3 μA/cm^2 in the presence and absence of NECA, respectively (n=4, P<0.001).

Nystatin permeabilization experiments were also used to determine the relative halide permeabilities of the basolateral ORCC in Calu-3 cells. Under conditions in which an equimolar Br^- gradient replaced the Cl^- gradient (Table 4.1), the ΔI_{sc} responses to nystatin and DIDS were reduced, though not significantly (P>0.05), to -97.8 ± 3.8 μA/cm^2 and 28.1 ± 1.1 μA/cm^2, respectively (n=4). When Cl^- ions were replaced by I^- ions the ΔI_{sc} response to nystatin was again insignificantly reduced (-84.8 ± 7.4 μA/cm^2, n=4, P>0.05), but the subsequent response to DIDS was reduced to 3.3 ± 0.3 μA/cm^2 (n=4, P>0.0001). Based on the DIDS-sensitive inhibition of the basolateral anion channels in Calu-3 cells, their halide permeability sequence is: Cl^- (1.00) ≥ Br^- (0.90) >> I^- (0.11).

Apical membrane permeabilization experiments were further used to investigate the current-voltage relationship of Cl^- permeation across the basolateral membrane of NHBE cells. Monolayers were bathed in symmetrical HCO_3^--free, choline solution (Table 4.1), and were clamped from -80 to 80 mV in 20 mV increments. The recordings, in Figure 4.4, show DIDS-sensitive current under control (Fig. 4.4A) and NECA-treated conditions (n=3, Fig. 4.4B). The current-voltage relationships, in Figure 4.4C, show that the DIDS-sensitive Cl^- current displays strong outward rectification, and is significantly stimulated by NECA (P<0.05). Similar experiments in Calu-3 cells were not possible as transepithelial resistances proved to be insufficient.

D) Adenosine signaling to basolateral anion channels:

Having established the localization and characteristics of the DIDS-sensitive anion channels activated by adenosine receptor stimulation, we investigated the signaling mechanisms involved. Our previous studies had demonstrated adenosine receptor expression and localization in Calu-3 cells (23), so the present study has only supplemented that information with data from NHBE cells. RT-PCR experiments revealed that NHBE cells express mRNA for A_{2A} and A_{2B} but not A_1 or A_3 receptors (Fig. 4.8). Adenosine receptor function and localization to either apical or basolateral membranes was characterized in transepithelial I_{sc} measurements in KHS (Fig. 4.9). These results show that adenosine-dependent I_{sc} can be nearly maximally stimulated by basolateral A_{2B} receptor stimulation, approximately half maximally stimulated by apical A_{2B} receptor stimulation and only slightly stimulated by basolateral A_{2A} receptor stimulation. Overall, this pattern is similar to that seen in Calu-3 cells.

Previous studies have shown that adenosine signaling is cAMP dependent in human airway epithelium (2). We have therefore investigated if cAMP dependent signaling is responsible for ORCC activation, in KHS. When Calu-3 cells are pre-treated with the PKA inhibitor, H-89 (bilateral, 10 μM), and NECA (bilateral, 10 μM) the I_{sc} response to DIDS (basolateral, 50 μM) is reduced by 80% to 2.7 ± 0.2 μA/cm^2 ($n=3$, $P<0.0005$, Fig. 4.5A), when compared to experiments in which there is no H-89 pre-treatment. Furthermore, forskolin (10 μM, bilateral), an adenylyl cyclase activator, stimulated a basolaterally DIDS sensitive I_{sc} that was similar to that activated by NECA ($n=4$, data not shown).

It has previously been shown that cAMP-dependent CFTR activation by adenosine receptors is A-kinase Anchoring Protein (AKAP) dependent (10). In order to investigate whether the same is true of basolateral Cl^- channel activation we used the stearated cell permeable form of the AKAP inhibiting peptide St-Ht31 and its AKAP inactive analog St-Ht31P, as a control. Calu-3 cells, in KHS, were pre-treated with either St-Ht31 (bilateral, 10 μM) or equimolar St-Ht31P, followed by NECA (bilateral, 10 μM). Under these conditions, the response to DIDS was reduced by 25% ($P<0.02$) in cells pre-treated with St-Ht31 (n=3) when compared to those pre-treated with St-Ht31P (n=3, Fig. 4.5B). Therefore, these experiments further support the involvement of cAMP in the activation of basolateral Cl^- channels and indicate that this activation may be partially AKAP dependent, though it is possible that disruption of AKAPs inhibits the DIDS response by subverting other channel activities.

Previous studies suggested that basolateral DIDS activates I_{sc} by stimulating an increase in $[Ca^{2+}]_i$ (1,14). We have therefore performed numerous experiments to test this hypothesis. First, we used the intracellular Ca^{2+} chelator BAPTA-AM in I_{sc} measurements. We found that application of BAPTA-AM (bilateral, 30 μM) to Calu-3 cells bathed in KHS induced a small decrease in I_{sc} of 2.4 ± 0.1 μA/cm^2 (n=6), which recovered over the next 30 minutes. There were no significant changes in the subsequent I_{sc} responses to either NECA (41.6 ± 1.4 μA/cm^2, n=3) or DIDS (18.7 ± 0.2 μA/cm^2, n=3) treatment. Second, we performed I_{sc} measurements on Calu-3 monolayers bathed in apical KHS, and basolateral Ca^{2+} free KHS (Table 4.1). It was previously shown that removal of basolateral Ca^{2+} does not disturb tight junctions, and allows for investigation of Ca^{2+}-independent responses (1,14,15). Indeed, in Calu-3 cells tight junctions remained

intact, as measured by R_T, and allowed for measurement of I_{sc} (Fig. 4.5D). In control experiments performed in bilateral KHS, the application of basolateral ionomycin (1 μM) induced a marked increase in I_{sc} (24.9 ± 5.7 μA/cm^2, n=4), when applied after NECA and DIDS (Fig. 4.5C). In Calu-3 cells bathed with apical KHS and basolateral Ca^{2+} free KHS, the responses to NECA (31.8 ± 7.3 μA/cm^2) and DIDS (14.2 ± 3.2 μA/cm^2) were unchanged (P>0.05), while the basolateral ionomycin response was completely absent until basolateral $CaCl_2$ was applied (2.5 mM, 21.1 ± 4.8 μA/cm^2, n=3, Fig. 4.5D). The carbachol response was also greatly inhibited by basolateral Ca^{2+} removal, from 151.3 ± 8.4 μA/cm^2 (n=6) to 16.6 ± 3.2 μA/cm^2 (n=3, P<0.0001). Experiments performed in bilateral Ca^{2+} free KHS resulted in greatly reduced R_T and therefore unreliable I_{sc}, though even then small but consistent and proportional responses to NECA and DIDS were observed (data not shown). Thus, our experiments show that the NECA and DIDS responses are Ca^{2+}-independent.

E) Basolateral ORCC and anion secretion:

Figure 4.6 shows typical I_{sc} responses to NECA and DIDS in HCO_3^--free and Cl^--free KHS in Calu-3 cells (Table 4.1). When HCO_3^- is removed from KHS, Calu-3 baseline I_{sc} decreases by 60% to 9.6 ± 1.0 μA/cm^2 (n=8, P<0.01), while in NHBE cells it does not change. Under these conditions the response to NECA in Calu-3 cells is not sustained. A robust initial peak in I_{sc} is followed by a return to baseline (Fig. 4.6A), resulting in an average increase of only 3.4 ± 0.8 μA/cm^2 (n=4). However, subsequent application of basolateral DIDS still induces a marked increase in I_{sc} of 9.4 ± 0.7 μA/cm^2 (n=4, Fig. 4.6A). When the order of drug addition was reversed (Fig. 4.6B), basolateral DIDS

increased I_{sc} by 2.0 ± 0.1 $\mu A/cm^2$ (n=4) and subsequent NECA application resulted in I_{sc} activation by 6.3 ± 0.8 $\mu A/cm^2$ (n=4), an effect that, despite the lack of an initial peak, was significantly greater then the response to NECA in the absence of DIDS ($P<0.05$). Lack of HCO_3^- ions in KHS did not affect I_{sc} responses in NHBE cells. NECA stimulated I_{sc} by 23.2 ± 1.4 $\mu A/cm^2$ (n=4) and subsequent DIDS application stimulated I_{sc} by 3.9 ± 0.6 $\mu A/cm^2$ (n=4), while DIDS did not affect baseline I_{sc}, and in its presence NECA induced an increase of 32.0 ± 2.9 $\mu A/cm^2$ (n=4).

When Cl^- is removed from KHS, baseline I_{sc} in Calu-3 cells is 16.7 ± 0.8 $\mu A/cm^2$ (n=3) while NECA stimulates the current by 27.4 ± 1.8 $\mu A/cm^2$ (n=3, Fig. 4.6C). Both of these values account for ~70% of the I_{sc} in normal KHS. This result is consistent with HCO_3^--free KHS experiments, which indicate that these cells primarily secrete HCO_3^-. Subsequent application of basolateral DIDS inhibits the I_{sc} by 10.4 ± 0.5 $\mu A/cm^2$ (n=3), likely due to its effects on basolateral Na^+-HCO_3^- cotransport. If DIDS is applied first in Calu-3 cells, it induces a small but significant rise in I_{sc} of 1.3 ± 0.1 $\mu A/cm^2$ (n=3, Fig. 4.6D). Subsequent application of NECA results in a reduced I_{sc} increase of 14.4 ± 0.7 $\mu A/cm^2$ (n=3), consistent with the notion that DIDS inhibits basolateral Na^+-HCO_3^- cotransport. In NHBE cells, Cl^- removal almost completely eliminates responses to adenosine receptor stimulation; NECA induces meager I_{sc} increases of 2.1 ± 0.7 $\mu A/cm^2$ (n=3) and 2.0 ± 0.5 $\mu A/cm^2$ (n=4), in the absence and presence of basolateral DIDS, respectively.

In order to further distinguish between Cl^- and HCO_3^- secretion we have performed simultaneous I_{sc} and radioisotopic flux measurements on Calu-3 cells in KHS. Figure 4.7A shows the averaged I_{sc} traces (n=8, the thickness of the line represents the SEM)

and the sample intervals used for counting (T_{40}-T_{80}). Figure 4.7B, compares the net $^{36}Cl^-$ flux rate (J^{Cl}_{net}) and the calculated net HCO_3^- flux rate ($J^{HCO_3}_{net}$) during each sample interval. This information is supplemented with measured unilateral $^{36}Cl^-$ flux data in Table 4.2; fluxes were measured n=4 times in each direction. These data confirm that the majority of baseline I_{sc} is HCO_3^- secretion (T_{40}), though the contribution of Cl^- to baseline varies greatly. Consistent with the findings from HCO_3^- free experiments (Fig. 4.6A), the application of bilateral NECA (10 μM) stimulates transient Cl^- secretion (Fig. 4.7, T_{50}) followed by sustained HCO_3^- secretion (Fig. 4.7, T_{60}). Unilateral flux data (Table 4.2) shows that the switch between Cl^- and HCO_3^- secretion occurs due to an initial stimulation of J^{Cl}_{BA} followed by an eventual "catch-up" by J^{Cl}_{AB}. Subsequent application of basolateral DIDS (Fig. 4.7, T_{70} and T_{80}) stimulates J^{Cl}_{net}, by decreasing J^{Cl}_{AB}, and reduces calculated HCO_3^- flux likely by inhibiting Na^+-HCO_3^- co-transport.

DISCUSSION

Our study shows that human airway epithelial cells possess basolateral Cl^- channels that are activated by A_2 adenosine receptors in a cAMP-dependent manner. Cell-attached patch-clamp experiments identified an outwardly rectifying chloride channel (ORCC) that was activated by adenosine receptor stimulation in Calu-3 cells. The channel properties, particularly its current-voltage relationship, were similar to those described previously (6,27,30). Similarly, Zhang *et al.* (31) have previously reported the presence of a cAMP-dependent outwardly rectifying DIDS-sensitive Cl^- conductance in patch clamp studies of NHBE cells. Our transepithelial I_{sc} measurements failed to detect any DIDS-sensitive anion channel activity in the apical membranes of either Calu-3 or NHBE cells, under control or adenosine receptor stimulated conditions. However, similar experiments demonstrated the presence of DIDS-sensitive adenosine receptor activated anion channel activity in the basolateral membranes of both cell types. These channels were 9-AC sensitive, but DNDS-insensitive and this finding was corroborated with apical membrane nystatin permeabilization studies, which also showed that DIDS-sensitive basolateral Cl^- current is outwardly rectifying in NHBE cells, and that this current can be stimulated by adenosine receptor activation. Similarly, Hwang *et al.* (11) have previously shown that the basolateral Cl^- conductance, in primary cultures of rat airway epithelium, is outwardly rectifying and cAMP-dependent. Thus there is strong evidence for the presence of basolateral ORCC in airway epithelial cells.

The halide permeability of the basolateral ORCC in Calu-3 cells ($Cl^- \geq Br^- >> I^-$) is characteristic of Eisenman selectivity sequence IV (29). It has previously been shown

that CFTR channel halide permeability is most consistent with Eisenman sequence III (12,24). However, it has been suggested that I^- may actually be more permeable than Cl^-, resulting in an Eisenman sequence I pattern of permeability ($I^- > Br^- > Cl^-$), but because I^- also blocks the pore it gives the apparent halide permeability sequence initially described (24). According to the Eisenman theory, the $Cl^-:HCO_3^-$ permeability ratio increases with the sequence number (29). Thus, the $Cl^-:HCO_3^-$ permeability ratio for CFTR (sequence I or III) is smaller than for ORCC (IV). In other words, the relative permeability of HCO_3^- is greater for CFTR than for ORCC, while the relative permeability of Cl^- is greater for ORCC than for CFTR.

Cobb *et al.* (2), have shown that adenosine receptors act through cAMP-dependent pathways that do not involve a change in $[Ca^{2+}]_i$ in Calu-3 cells. We have confirmed and extended this finding by showing that neither the adenosine nor the subsequent DIDS response is Ca^{2+}-dependent. These findings are significant as other studies have suggested that DIDS may stimulate anion secretion by raising $[Ca^{2+}]_i$ in T84 (1) and Calu-3 (14) cells. In contrast to our findings, Brayden *et al.* used T84 cells to show that when basolateral Ca^{2+} is depleted the basolateral DIDS-induced increase in I_{sc} is absent (1). These findings may represent differences between intestinal and airway epithelia. Studies by Ito *et al.* (14) have suggested that basolateral DIDS increases I_{sc} partially by increasing $[Ca^{2+}]_i$ in Calu-3 cells. Our results may differ because they studied the DIDS effect on baseline I_{sc} while we studied its effect on adenosine stimulated I_{sc}. Neither these studies, nor ours could address the Ca^{2+} issue directly because DIDS cannot be used in fluorescence studies (1). Perhaps the best argument in favour of our interpretation is the simplest - DIDS is a well known inhibitor of anion channels and our results can be

entirely explained by this mechanism of action; there is no need to ascribe new roles for extracellular DIDS in the form of effects on intracellular Ca^{2+}. Indeed, the data obtained by Ito *et al.* (14) can be re-interpreted, in light of our findings. They showed that Ca^{2+}-depletion and basolateral K_{Ca} blockers inhibit the basolateral DIDS response to a similar extent. They interpret this finding to mean that basolateral DIDS activates K_{Ca} by raising intracellular Ca^{2+}. However, it is more likely that in the absence of K_{Ca} activity, either through pharmacological block or by depletion of $[Ca^{2+}]_i$, the activity of any I_{sc} stimulating agent will be reduced because the driving force for anion secretion is reduced. In our experiments we pre-stimulated cells via a cAMP-dependent pathway, which is known to activate basolateral cAMP-dependent K^+ channels in Calu-3 cells (3). These cAMP-dependent K^+ channels likely compensated for the lack of K_{Ca} activity in the absence of $[Ca]_i$, and thus the basolateral DIDS response was conserved. This demonstrates that the DIDS-response is neither Ca^{2+} nor K_{Ca} channel dependent in Calu-3 cells. We further support our argument by showing that another anion channel blocker, structurally unrelated to DIDS, 9-AC, stimulates anion secretion when applied basolaterally. A similar explanation may be applicable to T84 cells, to account for the apparent Ca^{2+}-dependence of the basolateral DIDS effect (1), but this will require further experimentation and may be aided by the use of 9-AC. Thus, it is unlikely that basolateral DIDS alters $[Ca^{2+}]_i$; instead changes in $[Ca^{2+}]_i$ may alter the I_{sc} response to basolateral DIDS, under some conditions.

It has been shown previously that adenosine receptor signaling to CFTR in Calu-3 cells involves A Kinase Anchoring Proteins (AKAPs) (10). The results of our study show that AKAPs may also be involved in the activation of basolateral ORCC by adenosine

receptors. Further experimentation, particularly in patch clamp studies, will be necessary to prove this. However, binding to AKAPs may be an important clue in the determination of the molecular identity of this channel.

NHBE cells appear to secrete little or no HCO_3^-, under any conditions tested, and I_{sc} generated by these cells is due to Cl^- transport. In contrast, Calu-3 cells can secrete either Cl^- or HCO_3^-. HCO_3^- removal inhibits the majority of baseline I_{sc} and results in an adenosine response that displays an initial peak in I_{sc}, but no plateau. $^{36}Cl^-$ flux studies show that baseline I_{sc} is due to HCO_3^- secretion, the adenosine-dependent peak is due to Cl^- secretion, and the subsequent plateau is due to HCO_3^- secretion. In order to explain these results we propose the following model. CFTR channels are rapidly activated by adenosine resulting in an initial rise in J^{Cl}_{BA} (Table 4.2, T_{50}), whereas basolateral ORCC are activated after a delay, such that J^{Cl}_{AB} "catches up" shortly thereafter (Table 4.2, T_{60}). Opening of apical CFTR channels, which are more permeable to Cl^- then to HCO_3^-, results in Cl^- secretion. Subsequently, basolateral ORCC are activated, effectively redirecting Cl^- away from the apical membrane and causing it to be recycled across the basolateral membrane. At the same time, CFTR, which is more permeable to HCO_3^- than the basolateral ORCC, is now available to conduct HCO_3^-. This model explains our results, as well as those of others (5), that show that prolonged cAMP elevation in Calu-3 cells raises unidirectional Cl^- flux in both directions, resulting in no net secretion. However, while we have shown that this is likely due to activation of cAMP-dependent basolateral Cl^- channels, others suggested that this may be due to activation of cAMP-dependent electroneutral Cl^-:Cl^- exchange via basolateral anion exchangers (5). Either

explanation would be possible, but our studies also show that this effect is electrogenic indicating that it is mediated at least in part by ORCC.

Our model can also explain why Calu-3 HCO_3^- secretion dominates Cl^- secretion. Tamada *et al.* (25) showed that forskolin can activate apical membrane conductance so dramatically that HCO_3^- secretion can be easily sustained, despite the reduced permeability of CFTR to this anion. These authors postulated that preferential HCO_3^- secretion could be achieved if the basolateral NKCC cotransporters were inactive, in agreement with the fact that bumetanide has little effect on I_{sc}. Our data show that preferential HCO_3^- secretion can be achieved, even in the presence of NKCC activity, because Cl^- can be recycled across the basolateral membrane via ORCC.

Several other epithelia have been shown to secrete HCO_3^- preferentially over Cl^- (13,22), but the mechanisms by which this occurs are largely unknown. One of systems that has been well characterized is the pancreatic duct epithelium, which secretes Cl^- through apical CFTR channels, and then exchanges Cl^- for HCO_3^- through apical anion exchangers (22). On first inspection this model of HCO_3^- secretion as well as the one presented in the current study seems unnecessarily complicated. It would be far simpler for epithelia to express apical anion channels that are more permeable to HCO_3^- than Cl^-, and thus circumvent the need for apical anion exchangers or basolateral anion channels. However, currently there are no known anion channels that are more permeable to HCO_3^- then to Cl^-, and their existence is unlikely. An examination of anion isotherms (29) indicates that in order for an anion channel to be more permeable to HCO_3^- then to Cl^-, the pore would have to be very large, so large in fact that the channel may cease to display any selectivity. Therefore, epithelia may have been forced to evolve mechanisms,

such as the one described in the present study, to circumvent structural limitations of ion channels.

In our studies, when Cl^- is removed from the bathing solution basolateral DIDS inhibits adenosine-dependent I_{sc} in Calu-3 cells. This effect is likely due to inhibition of Na^+-HCO_3^- cotransporters, a known target for DIDS. Therefore, in KHS, the I_{sc} stimulated by DIDS in the presence of adenosine, reflects simultaneous basolateral Cl^- channel inhibition, which tends to raise I_{sc}, and basolateral Na^+-HCO_3^- cotransport inhibition, which tends to lower I_{sc}. In support of this argument, the inhibitory effect of basolateral DIDS in Cl^- free KHS (10.4 ± 0.5 $\mu A/cm^2$, Fig. 4.6C) is not statistically different from the inhibitory effect of basolateral DNDS in KHS (12.8 ± 1.6 $\mu A/cm^2$, n=3, Fig. 4.2D). Thus the DIDS stimulated I_{sc} increase (15.6 ± 2.0 $\mu A/cm^2$, Fig. 4.2A) measured in KHS after NECA addition, underestimates the contribution of basolateral Cl^- channels. A better measure of their contribution to I_{sc} is derived from experiments in which cells are pre-treated with NECA and DNDS prior to DIDS application (29.7 ± 1.1 $\mu A/cm^2$, Fig. 4.2D). This value agrees very closely with the directly measured J^{Cl}_{net} of 1.20 ± 0.13 $\mu Eq/cm^2h$ (32.2 ± 3.5 $\mu A/cm^2$, Fig. 4.7B, Table 4.2, T_{80}) stimulated by DIDS, in KHS and in the presence of NECA. Thus, these results suggest that in cells in HCO_3^- free KHS and pre-treated with NECA, DIDS should stimulate I_{sc} by ~30 $\mu A/cm^2$. However, I_{sc} measurements in HCO_3^- free KHS demonstrated increases of only 9.4 ± 0.7 $\mu A/cm^2$ (n=4). This indicates that Cl^- secretion in Calu-3 cells is HCO_3^- dependent, which can occur for a number of reasons: 1) HCO_3^- removal may depolarize the cell and therefore reduce the driving force for Cl^- secretion, 2) basolateral anion exchangers are involved, and 3) soluble adenylyl cyclase, which is HCO_3^- dependent and

is present in Calu-3 cells (unpublished data), is involved. In particular the possibility of anion exchanger involvement is interesting because it is also known to be DIDS-sensitive. The fact that there is a small, but statistically significant ($P<0.0001$) Cl^- absorption (T_{60}, Fig. 4.7B) in KHS occurring under short-circuit conditions after adenosine receptor stimulation, is consistent the involvement of such an anion exchanger. A finding supported in previous studies of Calu-3 cells that demonstrated the existence of a basolateral anion exchange process, likely mediated by Anion Exchanger 2, that contributed modestly to I_{sc} (17). This anion exchanger is a target for both DNDS and DIDS and thus its effects, like those of the Na^+-HCO_3^- cotransporter, can be factored out by sequential application of these drugs.

While our data clearly show that basolateral anion channels are important in determining the magnitude of Cl^- secretion, we also propose that they may be important in determining HCO_3^- secretion. Similar to the role of basolateral K^+ channels, the opening of basolateral anion channels may affect membrane potential. Specifically, the opening of basolateral anion channels is predicted to depolarize the cell thereby stimulating Na^+-HCO_3^- cotransporters and HCO_3^- secretion (25). However, because of limited pharmacological tools, and the lack of information regarding the molecular identity of ORCC, we could not directly address this issue.

In summary, this study not only expands our knowledge of epithelial cell function, but may also have important clinical implications. Our conclusions suggest that the ORCC may be an important modifier of the Cystic Fibrosis phenotype and that modulation of this channel may be a useful adjunct therapy for this or other illnesses characterized by abnormal airway epithelial anion secretion.

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TABLES

Table 4.1

Composition of Solutions used in Transepithelial Measurements (mM)

	*Normal			†HCO_3^- Free						
	KHS	Cl^- Free	Ca^{2+} Free	KHS	Low Cl^-	Br^-	Low Br^-	I^-	Low I^-	Choline
NaCl	117		117	117						
KCl	4.7		4.7	4.7	4.7					
$CaCl_2$	2.5			2.5	5					2.5
$MgCl_2$	1.2		1.2	1.2	1.2					1.2
KH_2PO_4	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	
$NaHCO_3$	24.8	24.8	24.8							
HEPES				10	10	10	10	10	10	10
Na gluconate		117			117		102.3		102.3	
K gluconate		4.7				4.7	4.7	4.7	4.7	
Ca gluconate		5				2.5	5	2.5	5	
Mg gluconate		1.2				1.2	1.2	1.2	1.2	
NaBr						126.7	14.7			
NaI								126.7	14.7	
Choline Cl										121.7
EGTA			1							
Glucose	11.1	11.1	11.1	11.1	11.1	11.1	11.1	11.1	11.1	11.1

*pH = 7.4 when bubbled with 5% CO_2/Balance O_2

†pH = 7.4 when bubbled with 100% O_2

Table 4.2

Unidirectional and Net Ion Fluxes Across Short-Circuited Calu-3 Monolayers

Sample	Treatment	J^{Cl}_{BA}	J^{Cl}_{AB}	J^{Cl}_{net}	I_{sc}	$J^{HCO_3}_{net}$	R_T
T_{40}	Control	-0.09±0.40	-0.08±0.75	0.00±0.54	0.91±0.04	0.91±0.04	183±6
T_{50}	NECA	4.08±0.24	1.84±0.17	2.23±0.19	2.07±0.05	-0.16±0.05	150±6
T_{60}	NECA	2.96±0.09	3.57±0.07	-0.61±0.07	2.33±0.05	2.94±0.05	153±4
T_{70}	NECA+DIDS	2.25±0.19	1.08±0.14	1.17±0.15	2.90±0.06	1.73±0.06	143±8
T_{80}	NECA+DIDS	1.94±0.10	0.74±0.18	1.20±0.13	2.54±0.08	1.34±0.08	151±3

Flux values and I_{sc} are in units of $\mu Eq/cm^2h$ and resistance (R_T) in Ωcm^2. NECA (10 μM) was added bilaterally, DIDS (50 μM) was added basolaterally. Each unidirectional flux was obtained from n=4 experiments, while net fluxes, I_{sc} and R_T were obtained from n=8 experiments.

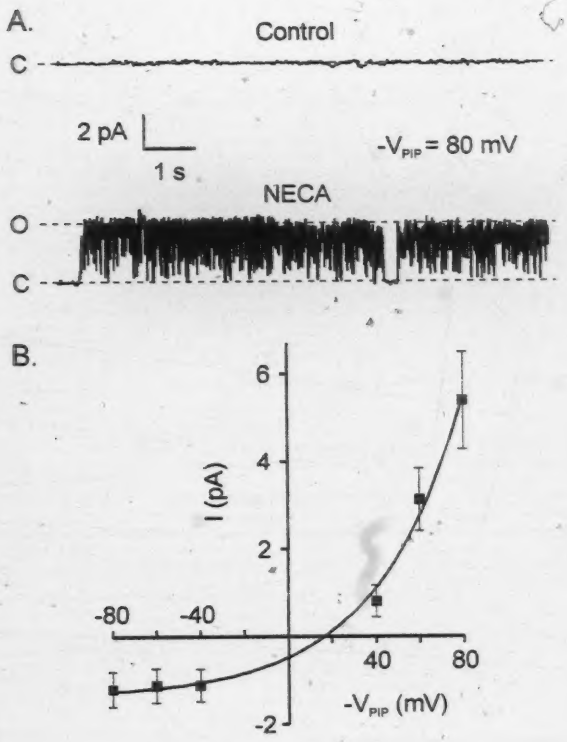
A.
Control
C
2 pA
1 s
-V_PIP = 80 mV
NECA
O
C
B.
6
4
2
-2
I (pA)
-80
-40
40
80
-V_PIP (mV)

Figure 4.1

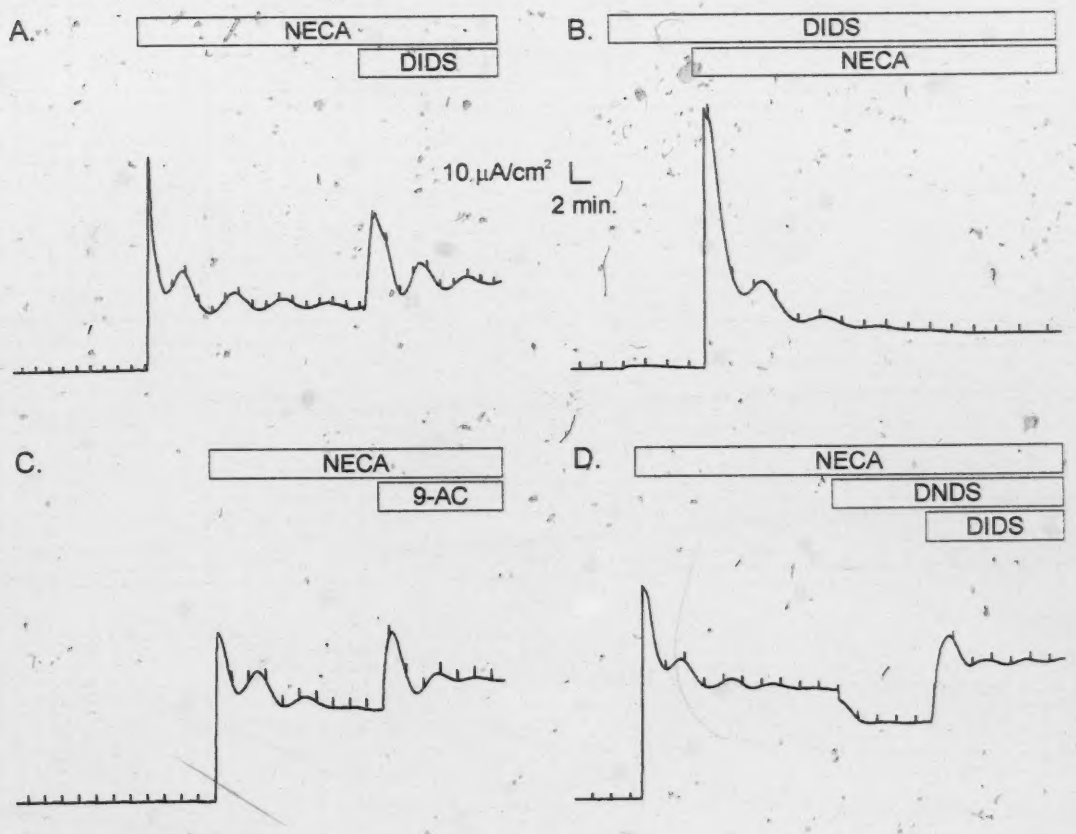
A.
NECA
DIDS
10 μA/cm²
2 min.
B.
DIDS
NECA
C.
NECA
9-AC
D.
NECA
DNDS
DIDS

Figure 4.2

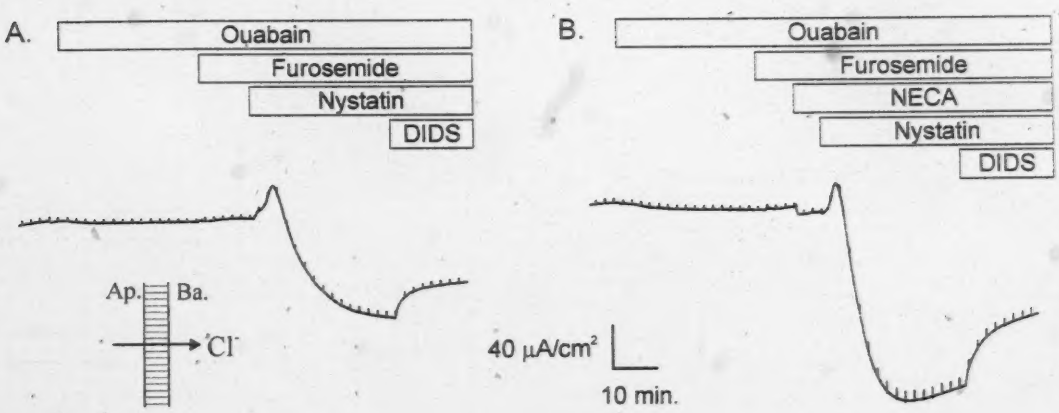
A.
Ouabain
Furosemide
Nystatin
DIDS
Ap.
Ba.
Cl-
B.
Ouabain
Furosemide
NECA
Nystatin
DIDS
40 μA/cm²
10 min.

Figure 4.3

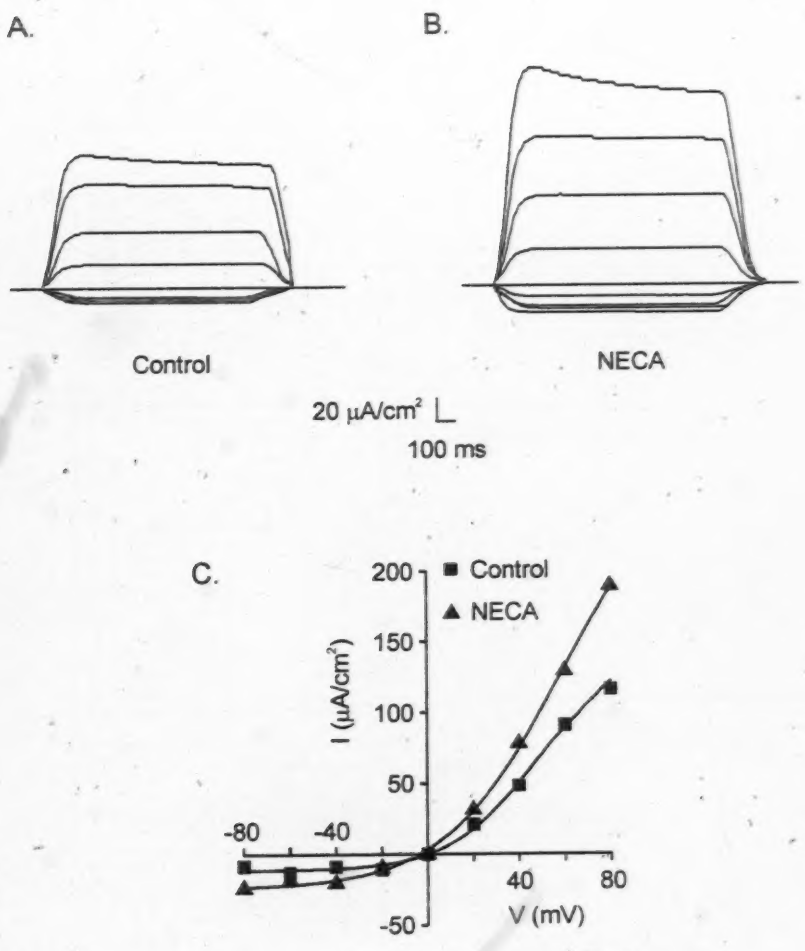
A.
B.
Control
NECA
20 μA/cm²
100 ms
C.
Control
NECA
I (μA/cm²)
200
150
100
50
-50
-80
-40
40
80
V (mV)

Figure 4.4

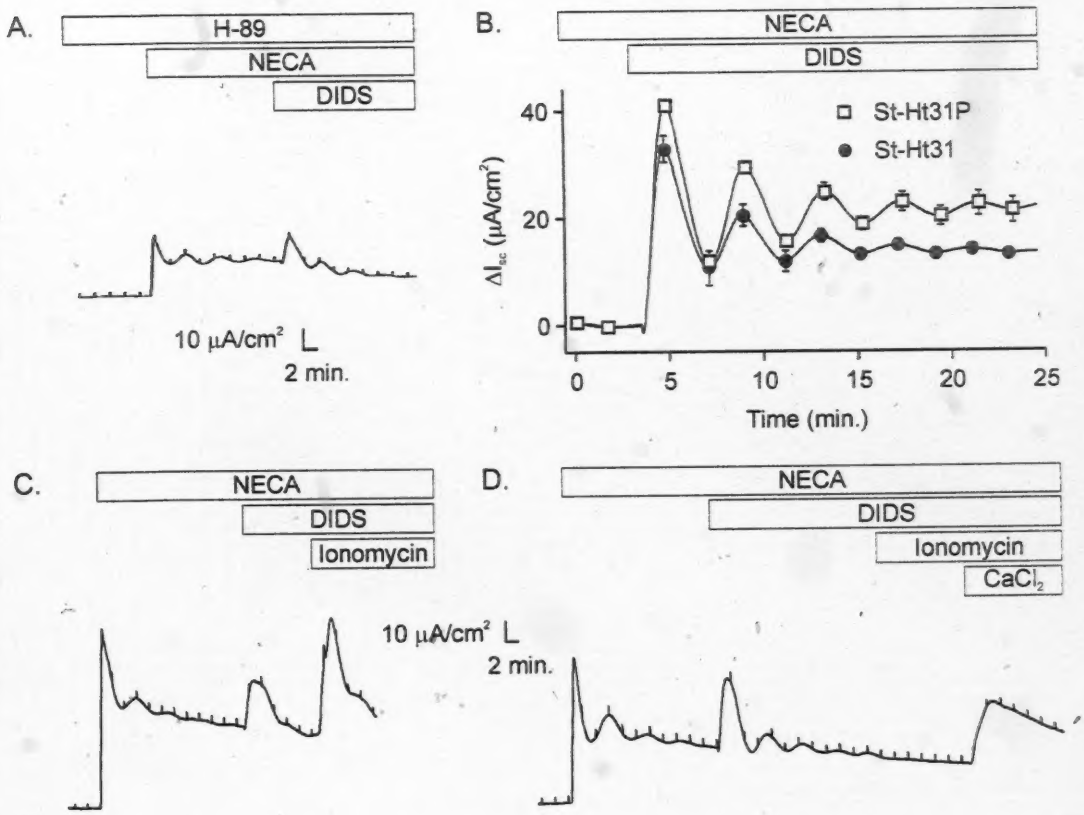
A.
H-89
NECA
DIDS
10 μA/cm²
2 min.
B.
NECA
DIDS
St-Ht31P
St-Ht31
ΔIsc (μA/cm²)
40
20
0
0
5
10
15
20
25
Time (min.)
C.
NECA
DIDS
Ionomycin
10 μA/cm²
2 min.
D.
NECA
DIDS
Ionomycin
CaCl2

Figure 4.5

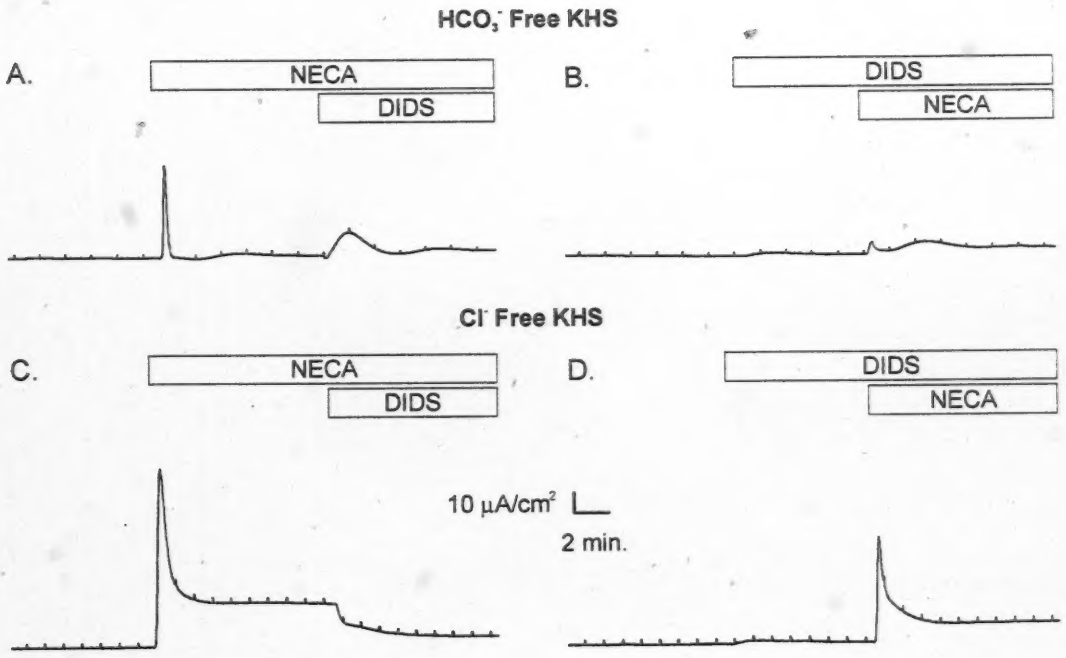
HCO3- Free KHS
A.
NECA
DIDS
B.
DIDS
NECA
Cl- Free KHS
C.
NECA
DIDS
D.
DIDS
NECA
10 μA/cm2
2 min.

Figure 4.6

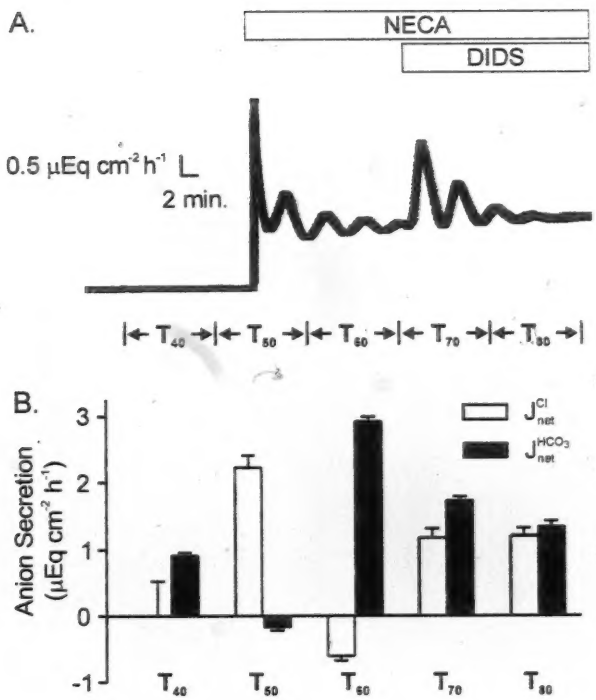
A.
NECA
DIDS
0.5 μEq cm⁻² h⁻¹
2 min.
T40
T50
T60
T70
T80
B.
Anion Secretion (μEq cm⁻² h⁻¹)
3
2
1
0
-1
J Cl net
J HCO3 net
T40
T50
T60
T70
T80

Figure 4.7

FIGURE LEGENDS

Figure 4.1

Adenosine-receptor mediated activation of an ORCC in Calu-3 cells. ***A*:** Typical traces, obtained in 160 mM Tris Cl, 30 mM sucrose solution, showing ORCC activity in a cell-attached patch before and after NECA (10 μM) treatment. The recordings are representative of ten experiments, 4 of which displayed channel activity, and the channel closed (C) and open (O) states are shown. ***B*:** The current-voltage relationship corresponds to the experiment shown in panel ***A*** after NECA treatment. Values are expressed as means ± SD.

Figure 4.2

The effect of NECA, and basolateral anion channel blockers on I_{sc} in Calu-3 cells. Transepithelial I_{sc} measurements were performed in KHS in the presence of apical amiloride (10 μM). ***A***: Representative recording (n=8) showing the activation of I_{sc} by bilateral NECA (10 μM), and subsequent basolateral DIDS (50 μM). ***B***: The effect of basolateral DIDS on baseline I_{sc}, and potentiation of the subsequent NECA response (n=3). ***C***: Basolateral 9-AC (1 mM) application stimulates I_{sc} to a similar extent as basolateral DIDS, in the presence of NECA (n=3). ***D***: Basolateral DNDS (3 mM) inhibits I_{sc}, when applied after NECA; the subsequent basolateral DIDS response is potentiated (n=3).

Figure 4.3

Permeabilization of the apical membrane with nystatin in Calu-3 cells reveals a basolateral Cl^- conductance that can be stimulated by NECA and is inhibited by DIDS. I_{sc} measurements were performed in the presence of an apical (HCO_3^- Free KHS, Table 4.1) to basolateral (HCO_3^- Free, Low Cl^- solution, Table 4.1) Cl^- gradient (129.1 mM:17.1 mM). Cells were pre-treated with basolateral ouabain (1 mM), to inhibit the Na^+/K^+-ATPase, and basolateral furosemide (1 mM) to inhibit Na^+-K^+-$2Cl^-$ cotransport. Representative recordings are shown from control (***A***, n=4) and NECA treated (***B***, bilateral, 10 μM, n=3) monolayers exposed to apical nystatin (90 μg/ml) and subsequent basolateral DIDS (50 μM).

Figure 4.4

Permeabilization of the apical membrane with nystatin in NHBE cells reveals the presence of DIDS-sensitive ORCC in the basolateral membrane. Current measurements were performed in symmetrical HCO_3^--free choline Cl^- solution (Table 4.1), in cells permeabilized with apical nystatin (90 μg/ml), and clamped from -80 to 80 mV in 20 mV increments. Once currents had stabilized after nystatin permeabilization, basolateral DIDS (50 μM) was applied and further recordings were made. Representative DIDS-sensitive current recordings from control (***A***) and NECA (bilateral, 10 μM, ***B***) treated monolayers (n=3) are shown. ***C***: Current-voltage relationships for traces shown in panel ***A***.

Figure 4.5

Adenosine receptor mediated activation of basolateral anion channels is Protein Kinase A (PKA) and A-Kinase Anchoring Protein (AKAP) dependent, but Ca^{2+}-independent in Calu-3 cells. Experiments were performed on monolayers pre-treated with apical amiloride (10 μM). ***A***: In cells, bathed in bilateral KHS, and pre-treated with the PKA inhibitor, H-89 (bilateral, 10 μM), the bilateral NECA (10 μM) and basolateral DIDS (50 μM) responses were greatly reduced (n=3). ***B***: Calu-3 monolayers, bathed in bilateral KHS, were pretreated with either the AKAP neutralizing peptide St-Ht31 (bilateral, 10 μM), or an equimolar amount of AKAP-inactive St-Ht31P as a negative control. I_{sc} experiments were then performed by adding bilateral NECA (10 μM, not shown), followed by basolateral DIDS (50 μM). The averages of three of each experiment are shown. The values expressed are means ± SEM. ***C:*** In cells bathed with bilateral KHS, and pre-treated with bilateral NECA (10 μM) and basolateral DIDS (50 μM), there is a significant response to subsequent application of basolateral ionomycin (1 μM). ***D:*** In cells bathed with apical KHS, and basolateral Ca^{2+}-free KHS, the responses to bilateral NECA (10 μM) and basolateral DIDS (50 μM) are unchanged, but application of basolateral ionomycin (1 μM) has no effect; subsequent application of $CaCl_2$ (2.5 mM, basolateral) stimulates I_{sc}.

Figure 4.6

The effect of NECA and DIDS on I_{sc} in Calu-3 cells in HCO_3^-- and Cl^--free solutions. All monolayers were pre-treated with apical amiloride (10 μM). Representative recordings are shown from experiments performed by either applying bilateral NECA (10 μM)

followed by basolateral DIDS (50 μM) (***A***, ***C***, n=4) or by applying basolateral DIDS followed by bilateral NECA (***B***, ***D***, n=3).

Figure 4.7

Simultaneous $^{36}Cl^-$ flux and I_{sc} measurements demonstrate the relative contributions of HCO_3^- and Cl^- to Calu-3 anion secretion. Experiments were performed in KHS on monolayers pre-treated with apical amiloride (10 μM). ***A***: The averaged transepithelial I_{sc} from the n=8 experiments during which radioisotopic flux measurements were performed. The data are expressed as a mean ± SEM, where the thickness of the trace represents the SEM. The sampling periods for radioisotopic flux measurements, designated T_X (where X is the time in minutes), are shown in relation to the points at which bilateral NECA (10 μM) and basolateral DIDS (50 μM) were added. ***B***: Measured $^{36}Cl^-$ net flux ($J^{Cl}_{net} = J^{Cl}_{BA} - J^{Cl}_{AB}$) and calculated HCO_3^- flux ($J^{HCO_3}_{net} = I_{sc} - J^{Cl}_{net}$) are shown for each sampling period. Unilateral fluxes and other relevant data are summarized in Table 4.2. All values are expressed as means ± SEM.

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SUPPLEMENTARY MATERIALS

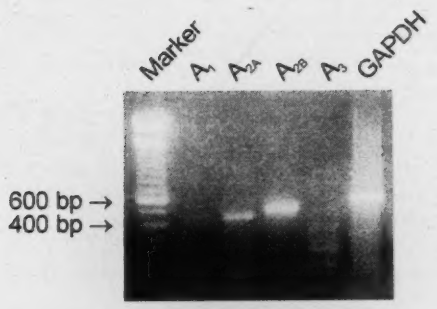

Figure 4.8

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for NHBE Cell Adenosine Receptors. A portion (3×10^6) of NHBE cells harvested from T75 flasks were used for RNA purification using the Qiagen RNeasy kit (Qiagen). First-strand cDNA was synthesized by reverse transcription of the RNA using Superscript II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers (200 ng). Thereafter, PCR was performed using the following sets of primers (Invitrogen, from 5' to 3'): A_1 (GenBank™ accession number L22214) forward nucleotides 603-623, reverse 847-826; A_{2A} (U40771) forward 546-569, reverse 995-972; A_{2B} (X68487) forward 447-466, reverse 958-938; A_3 (L22607) forward 353-376, reverse 793-770. In addition to the primers designed to amplify sequences of interest, reactions with glyceraldehyde-3-phosphate dehydrogenase specific primers were run in all rounds of PCR reactions to serve as internal positive controls: GAPDH (accession number M33197) forward 212-235, reverse 806-786. PCR was performed using the hot-start method, where one tenth of the reverse transcription reaction was combined with 1 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH

8.4), 2.5 U of Taq polymerase, and autoclaved distilled water to a final volume of 20 µl. After 5 min at 94°C, amplifications proceeded under the following conditions: 30 cycles (94°C, 45 s; 55°C, 30 s; 72°C, 90 s) with a final elongation period at 72°C for 7 min. PCR products and Marker (100 bp DNA ladder; Invitrogen) were separated and visualized by electrophoresis on ethidium bromide-stained 1.5% agarose gels. The expected sizes (bp) of the PCR products were: 245 (A_1), 450 (A_{2A}), 512 (A_{2B}), 441 (A_3), and 595 (GAPDH); arrows indicate the positions of the marker fragments. To confirm their identities, all RT-PCR products were sequenced by the University of Alberta DNA Sequencing Core Facility. NHBE cells express transcripts for A_{2A} and A_{2B} but not A_1, and A_3 receptors.

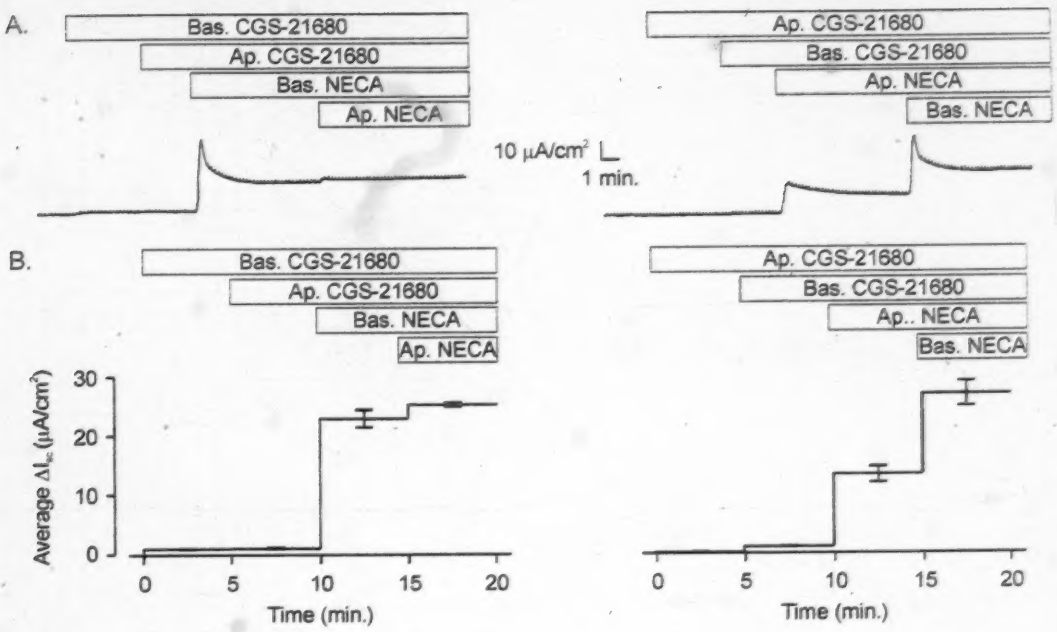

Figure 4.9

Functional characterization of polarized adenosine receptor distribution in NHBE cells. Cells were bathed in bilateral KHS, pre-treated with apical Amiloride (10 μM), and I_{sc} was recorded continuously. CGS-21680 (1 μM), was used to selectively activate A_{2A} receptors, while the sequential application of NECA (10 μM), was used to activate A_{2B} receptors. Representative recordings from these experiments in NHBE cells are shown in Figure 4.9A, while the statistical analyses derived from these recording are shown in Figure 4.9B. When CGS-21680 was applied to the basolateral membrane it induced a small but statistically significant increase in I_{sc} of 0.9 ± 0.1 μA/cm^2 (n=4, P<0.01). In contrast, apical application of CGS-21680 failed to elicit a response in the I_{sc} (n=4). NECA was applied in the presence of CGS-21680, and was found to have a much more significant effect on the I_{sc}. This effect was greater when NECA was applied only to the basolateral membrane rather than only to the apical membrane. Basolateral NECA

stimulated the I_{sc} by 21.1 ± 1.4 μA/cm^2 (n=3), which was only slightly increased by an additional 2.2 ± 0.3 μA/cm^2 (n=3) with the subsequent application of apical NECA. When the order of addition was reversed, apical NECA stimulated the I_{sc} by 11.7 ± 1.3 μA/cm^2 (n=3), and subsequent application of basolateral NECA induced an additional increase of 13.1 ± 2 μA/cm^2 (n=3). All values are expressed as means ± SEM. Abbreviations: Ap., Apical; Bas., Basolateral; Bi., Bilateral.

CHAPTER 5

General Discussion

Our studies on the regulation of airway epithelial ion transport by adenosine have employed three models of human airway epithelium. These cells were cultured *in vitro*, under conditions that attempt to mimic *in vivo* conditions, as closely as possible. In particular, whenever possible, the cells were grown at an air liquid interface, with media applied to the basolateral, but not the apical aspect of monolayers. This technique has been shown to be vital for differentiation of *in vitro* cultures of airway epithelia (25,26,32,49). Thus, we expect that the use of three well differentiated airway epithelial cell models should produce experimental data that is likely to be consistent with native airway epithelium.

A549 cells are a model of human type II alveolar pneumocytes. These cells were derived from an explanted human lung adenocarcinoma, established as a cell line in 1972 and are available from the American Type Culture Collection (Rockville, MD). They contain lamellar cytoplasmic inclusions necessary for surfactant production, which are a characteristic marker of type II pneumocytes (36). They form confluent monolayers, but they are relatively "leaky", with a low R_T and are therefore unsuited for transepithelial measurements of I_{sc} or V_T. However, studies have shown that A549 monolayers are highly polarized and do constitute a valid model of type II alveolar pneumocytes (20). Furthermore, because A549 cells do not express CFTR, they represent a convenient model for the study of CFTR-independent ion transport (18).

Calu-3 cells are a model of human serous submucosal gland epithelium. Similar to A549 cells, they were derived from an explanted human lung adenocarcinoma, established as a cell line in 1975 and are also available from the American Type Culture Collection. However, unlike A549 cells, Calu-3 cells form monolayers with high R_T,

display cAMP-dependent anion secretion under short-circuit conditions and express high levels of CFTR, all of which are characteristics of native submucosal epithelium (18,54). Furthermore, these cells secrete several markers of submucosal epithelium including lysozyme, secretory leukocyte protease inhibitor and lactoferrin (18). These cells, in particular, have been touted as an excellent cell-line model of airway epithelium (19,26).

Primary cultures of normal human bronchial epithelium (NHBE cells) were grown under conditions that have been shown to produce monolayers with ultrastructures that are essentially identical to that of native epithelium (25,63). The cells are obtained from BioWhittaker (San Diego, CA) as frozen passage 1 stocks and used at up to passage 3. Because these cells are not transformed, they are likely to provide the most accurate representation of native airway epithelial function of the three models systems used in the present studies. However, because these cells are derived from surface bronchial epithelium, they are likely to respond differently then native alveolar and submucosal epithelium, necessitating the use of the A549 and Calu-3 cell lines.

AIRWAY EPITHELIAL ADENOSINE RECEPTORS

The first report of airway epithelial anion secretion stimulated by adenosine was in 1986 (51). Since that time numerous studies have confirmed that adenosine stimulates anion secretion in various models of airway epithelia, but have diverged when it comes to the identity of the adenosine receptors responsible for this effect (3,4,6,28,29,34,38,45,53). In primary cultures of human nasal epithelium, and in two bronchial epithelial cell lines, BEAS39 and CF/T43 cells, Lazarowski *et al.* (34)

demonstrated an adenosine analogue rank order of potency consistent with A_2 receptor stimulated anion secretion, which was cAMP-dependent; they also suggested that adenosine receptor distribution does not differ between the apical and basolateral membranes. At the same time, other studies performed on transformed airway epithelial cell lines – 9HTEo$^-$ (normal adult tracheal epithelium), 56FHTEo$^-$ (normal fetal tracheal epithelium), CFNPE9o$^-$ (CF nasal polyp cells) and CFPEo$^-$ (CF submucosal gland cells), showed that A_1 receptors are responsible for stimulating Cl^- and K^+ conductances, and that this occurs through a Ca^{2+}-dependent pathway (22,53). Perplexingly, studies by Chao *et al.* (3), in CFPEo$^-$ cells demonstrated that it was A_2, not A_1, receptors that were stimulating Ca^{2+}-dependent ion channels; while studies by McCoy *et al.* (38) in 9HTEo$^-$ cells, CFSMEo$^-$ cells (transformed CF submucosal airway epithelium) and primary cultures of nasal polyp epithelium demonstrated that the A_1 receptor antagonist DPCPX functions to stimulate CFTR current, even in CF cells, presumably by preventing A_1-dependent cAMP attenuation. Studies in Calu-3 cells have suggested that adenosine activation of CFTR is cAMP-dependent and likely involves A_{2B} receptors (6,28,29). Part of the confusion surrounding this issue can be attributed to recent findings showing that some xanthines, particularly the A_1 antagonist – DPCPX, may actually stimulate CFTR activity directly (8,27).

The controversy over the exact nature and function of adenosine receptors in human airway epithelium has important implications for CF treatment. If adenosine stimulates anion secretion through an A_1 (or A_2) Ca^{2+}-dependent process, it may play an important role in CF therapy; Ca^{2+}-dependent anion secretion is conserved and may even be upregulated in individuals with CF (see chapter 1, section 2.1). Alternatively, if

adenosine stimulates anion secretion through an A_2 cAMP-dependent process, its application to CF therapy is less likely, since CFTR mutation results in defective cAMP-dependent anion secretion. However, recent studies by Clancy *et al.* (4), have shown that activation of cAMP-dependent A_{2B} receptors may be of benefit in the treatment of individuals with a subset of CFTR mutations that display normal trafficking to the plasma membrane, such as the R117H mutant.

Another significant problem, encountered in studies focused on the characterization of adenosine receptor distribution, is that adenosine appears to be able to diffuse across epithelia (6,51). This occurs despite the formation of confluent monolayers with high R_T, and despite the hydrophilic nature of adenosine. In preliminary studies, we noticed that adenosine stimulates I_{sc} to the same extent whether applied from the apical, basolateral or bilateral aspect of monolayers. This implied that either adenosine receptor distribution is not polarized or that adenosine is getting across the "tight" epithelium. The latter explanation was favoured in subsequent experiments using the adenosine analogs – NECA and CGS-21680, which produced polarized I_{sc} responses. These results can be explained by facilitated transcellular transport of adenosine, but not its derivatives NECA and CGS-21680. Thus, the impermeability of these drugs was exploited in order to functionally characterize adenosine receptor distribution.

Our studies showed that upper airway epithelia are regulated by adenosine via A_{2A} and A_{2B} receptors. Experiments in Calu-3 and NHBE cells were consistent with a cAMP-dependent and primarily A_{2B} receptor mediated response, which exhibited differential polarization in the two cell types. A minor A_{2A} receptor dependent response was also apparent in the basolateral membranes of these cells. It is possible that the receptor

expression and distribution pattern differs in other regions of the airway as suggested by other studies. However, our use of primary bronchial cultures is likely to reflect the function of native epithelium. Furthermore, bronchial surface epithelium and submucosal serous cells are particularly relevant to the pathology of CF.

In contrast to upper airway epithelium, our studies in A549 cells revealed that ion channel regulation in this cell type is A_1 receptor dependent. Furthermore, this regulation appears to be Ca^{2+}-dependent, for a number of reasons: 1) Ca^{2+}-dependent K^+ channels (IK-1) are activated by adenosine, 2) modest increases in $[Ca^{2+}]_i$ were seen in fluorescence studies using Fura-2 (unpublished data), and 3) these receptors are usually coupled to G_i-proteins, whose stimulation is often associated with increased $[Ca^{2+}]_i$. The A_{2A} and A_{2B} receptor subtypes are also expressed in this cell line, and may be coupled to cAMP via G_s-proteins, as would be consistent with the pattern of adenosine receptor expression and function in airway epithelia. It is possible that these cells do not display an apparent whole-cell current response to A_2 receptor stimulation because they lack cAMP-dependent ion channel targets, such as CFTR (18).

AIRWAY EPITHELIAL ADENOSINE TRANSPORT AND METABOLISM

Our model of adenosine homeostasis in airway epithelia is shown in Figure 3.7. Three factors have been identified in our studies as being important for determining adenosine-dependent ion transport: 5'-nucleotidase, adenosine kinase and nucleoside transporters. Inhibitors of all three factors exist, which, when applied result in inhibition, stimulation and stimulation of adenosine-dependent ion transport, respectively. Furthermore, while

ion transport was used as a functional marker of adenosine efficacy in our studies, this mediator has been shown to have other potentially beneficial effects in the airway, such as the stimulation of ciliary beating (41). Thus, if it is shown that adenosine receptor modulation can be of benefit in the treatment of airway disease, each of these three factors may represent novel therapeutic targets. However, we have focused on the role of nucleoside transporters in the airways, in particular.

Our studies indicate that equilibrative nucleoside transport processes exist in both the apical and basolateral membranes of airway epithelia. In intestinal epithelium, the expression of apical concentrative and basolateral equilibrative transporters appears to facilitate nucleoside absorption (64). In the airway the purpose of the pattern of transporter distribution is not as obvious, but one of its consequences may be an equalization of transepithelial nucleoside concentrations (discussed above). Thus, the permeability of any nucleoside derived pharmacological agent must be taken into account. This is particularly true of ATP, and its analogs, which are currently being investigated as potentially therapeutic agents in the treatment of CF (48). Inhaled ATP analogs stimulate apical P_2-receptors, which activate anion secretion via apical Ca^{2+}-dependent Cl^- channels (33,50). Our studies indicate that ATP will be broken down to adenosine, which may cross the epithelium, likely via nucleoside transporters and therefore gain access to the submucosa. This may present a problem as submucosal adenosine can stimulate smooth muscle A_{2B} receptors and lead to bronchoconstriction, under some conditions (17,44). Thus, our studies justify the use of ATP analogs that cannot be broken down to A_{2B} receptor agonists. Clinically, uridine 5'-triphosphate (UTP) has been investigated as an alternative to ATP in the treatment of CF (48). This

appears to be an adequate resolution to the problem, as this mediator is metabolized to uridine, which is a universal nucleoside transporter substrate, but has no effect on any of the known P_1-receptors. However, because the action of UTP is too brief to be clinically useful (24), longer acting ATP analogs will need to be sought. The epithelial transport, metabolism and receptor activity profiles of these analogs and their breakdown products will need to be characterized, before they can be used clinically.

One of the most clinically relevant models of nucleoside homeostasis is the heart, where the equilibrative nucleoside transport inhibitors, dipyridamole, dilazep and draflazine, are used as vasodilators and cardio-protective agents. In cardiomyocytes, the adenosine concentration gradient is normally inwardly directed, such that inhibition of nucleoside transport elevates extracellular adenosine concentrations leading to receptor stimulation in coronary vessels and vasodilation (11,12). In contrast, hypoxia induces intracellular ATP breakdown and conversion to adenosine. This appears to be particularly dependent on reduced adenosine kinase activity, which is inhibited under hypoxic conditions (9). Thus, the adenosine gradient becomes reversed, resulting in adenosine efflux from cardiomyocytes. This plays a physiologically protective role, as released adenosine then promotes coronary vasodilation and a slowing in the heart rate, through P_1-receptor dependent mechanisms. It is unclear why nucleoside transport inhibitors promote extracellular adenosine accumulation under these conditions. This may be explained by suggestions that nucleoside transport inhibitors primarily target coronary vascular endothelium, which take up and metabolize adenosine during hypoxia, while cardiomyocytes secrete it (42,58). It may be helpful for future studies to compare *es* and *ei* transporter activities in the cardiomyocytes and the endothelium, which display

differential sensitivity to inhibition by these drugs. A similar response to hypoxia is seen in the intestinal epithelium where adenosine efflux is promoted, which then induces receptor-mediated anion secretion resulting in the clinical phenomenon of ischemic diarrhea (37).

There has been relatively little investigation into airway epithelial ion transport under hypoxic conditions, though this would be clearly relevant to the pathology of many pulmonary illnesses characterized by abnormal ASL. For example, in acute asthmatic attacks there is sudden, global hypoxia; in CF, thick mucus plaques have been shown to induce focal epithelial hypoxia (61); finally, in adult and neonatal respiratory distress syndromes, fluid accumulation results in alveolar hypoxia. It is quite likely that intracellular adenosine accumulation and efflux from airway epithelia, similar to that seen in the heart and intestine, could occur via equilibrative nucleoside transporters, and alter ion transport under these conditions. This may account for a portion of the increased adenosine concentration seen in the bronchoalveolar lavage fluid of asthmatics (15). Most investigation linking hypoxia and ion transport in airway epithelium has occurred in alveolar pneumocytes, where changes in channel and ion transporter expression are the focus (for review see 5). However, at least one study has shown that hypoxia also induces acute changes in alveolar epithelial ion transport, through an unknown mechanism, which is rapidly reversed by β-agonists (59). Thus, because adenosine signaling pathways are important for the regulation of ion transport in airway epithelia and are known to be sensitive to hypoxia, further studies will be required in order to determine what role adenosine plays under these conditions.

Our studies show that nucleoside transport activity is an important determinant of extracellular adenosine concentrations, however, we have not directly shown that nucleoside transporters are true regulators of adenosine. Regulation would imply that: 1) nucleoside transporter activity can be modulated by the epithelial cells, and 2) that changes in nucleoside transporter activity are paralleled by changes in adenosine receptor activity. Regarding the first stipulation, the most frequently reported regulation of equilibrative transporter activity is by PKC, and has been shown to occur in various cell types (7,10,43). However, it remains unclear as to how this regulation is occurring, or even if it is stimulatory or inhibitory. Other studies have implicated various signaling molecules such as cAMP, cGMP, nitric oxide, insulin and D-glucose in the regulation of nucleoside transport activity (1,2,40,56). Thus, it is clear that nucleoside transporters are regulated, though the mechanisms of this regulation require further study and likely differ between cell-types. Regarding the second stipulation, our studies suggest that nucleoside transport activity is inversely correlated with adenosine receptor activity. In A549 cells the nucleoside transport activity (Fig. 2.10A, B) in both the apical and basolateral membranes is almost an order of magnitude greater then the nucleoside transport activity in Calu-3 cells (Fig. 3.3). At the same time, baseline whole-cell current in A549 cells is completely adenosine receptor independent, while in Calu-3 cells much of the baseline whole-cell current can be inhibited by adenosine receptor or 5'-nucleotidase antagonists. Of course, as we have shown, other factors also determine the extracellular concentration of adenosine and, therefore, these findings can only suggest a correlation between nucleoside transporter activity and adenosine regulation. This suggestion is appealing and worthy of further investigation because 1) nucleoside transport into the cell appears to be

the rate-limiting step in adenosine metabolism, and 2) potential upstream points of regulation are also likely to influence signaling by other mediators such as ATP. Thus, further investigation of nucleoside transport in the airway epithelium and other tissues is well justified, particularly considering that specific pharmacological agents are not only readily available, but also approved for clinical use.

THE ION CHANNEL TARGETS OF ADENOSINE SIGNALING

Numerous transporters and channels work together to mediate vectorial ion transport in airway epithelia. However, we have chosen to focus on ion channels, for a number of reasons. First, there are more techniques available for the study of ion channels, in particular the patch-clamp, which is extremely powerful but rarely applicable to the study of transporters. Second, there is a great diversity of ion channels, such that they are often specific to a particular tissue or cell type. Therefore, there is the potential to develop channel subtype specific pharmacological agents that will target specific problems or tissues, without causing widespread side effects. Third, channels have much greater turnover rates (10^7-10^8 ions/s) then transporters (10^2-10^4 ions/s). This has extremely important consequences for all cells. For example, a typical cell with a volume of 10^{-10} ml and a 100 mM intracellular K^+ ion concentration (~10^9 ions) could be drained of all its K^+ by a single channel (with a turnover rate of 10^8 ions/s) in 10 seconds. Thus, considering that cells usually contain numerous channels, they have the ability to cause profound and rapid changes in ion concentrations and membrane potentials. This ability is exploited by excitable tissues to propagate rapid electrical signals. However, it requires

that ion channel activity in all cells be regulated very tightly. Thus, the mechanisms of ion channel regulation are of great importance. This thesis focuses on the regulation of ion channels by adenosine, an important endogenous mediator that is always present at the surface of airway epithelial cells to some extent, and is therefore involved in the minute by minute control of these channels.

In A549 cells, intermediate conductance Ca^{2+} dependent K^+ (IK) channels were identified as an important target for adenosine. The discovery of IK channels in this cell type was a significant finding. Very little is known about the types of K^+ channels expressed in human alveolar epithelia, since the majority of studies have been performed in the rat (for review see 46). Only one other study has examined Ca^{2+}-dependent K^+ channel activity in type II pneumocytes. Ridge *et al.* (52) showed that single-channel activity characteristic of the biophysical properties of BK channels was frequently observed in excised inside-out patches of A549 cells. Interestingly, they also commented on the presence of an intermediate conductance channel that had biophysical properties consistent with IK and was almost always seen in the same patches as the BK channels. In light of the findings presented in this thesis, and the cloning of the human IK-1 channel (31), it appears likely that the secondary channel observed by Ridge *et al.* (52) was an IK. Interestingly, in studies performed by O'Grady *et al.* (46) after the publication of our data, neither BK nor IK channel mRNA could be detected in rat alveolar epithelial cells via RT-PCR.

Our data show that IK channels are responsible for most of the adenosine effect on whole-cell current in A549 cells. Presumably, this regulation by adenosine is Ca^{2+} dependent, as would be characteristic for regulation via the A_1 receptors shown to

mediate this response. Because IK channels are more sensitive to $[Ca]_i$ then BK channels, it may be that changes in this intracellular secondary messenger were not large enough to influence BK activity. Indeed, the increase in whole-cell conductance stimulated by adenosine (141 ± 14 pS/pF) was much less than that elicited by EBIO (468 ± 35 pS/pF), a drug expected to maximally stimulate IK channels; suggesting that adenosine is a relatively weak stimulus.

The activation of IK channels by adenosine may have variable effects on transepithelial ion transport. Typically, adult alveolar type II cells primarily absorb Na^+ and fluid, though ENaC activity was not observed in our whole-cell patch clamp studies. possibly due to submerged cell culture conditions known to prevent expression of this channel (see chapter 1, section 2.2). Since K^+ channels are usually localized to the basolateral membrane in epithelia, it can be predicted that the identified IK channel is as well. In this case, activation of this channel would promote Na^+ and fluid absorption by hyperpolarizing the cell and therefore promoting passive Na^+ uptake across the apical membrane, which is the rate-limiting step in this process. Alternatively, other studies have demonstrated anion transport in A549 cells (16). Our studies identified DIDS-sensitive anion channels, which may mediate a portion of anion secretion. This process could be potentiated by basolateral IK channel activation, though these anion channels did not appear to be a target of adenosine signaling, but were constitutively active. Of course, apical IK-1 localization, and the possibility that it plays a role in K^+ secretion cannot be ruled out.

Another novel target for adenosine signaling identified in airway epithelium were the basolateral anion channels. The existence of these channels has been suggested in a

number of previous studies, beginning as early as 1989 (60). In particular, Wine *et al.* (62) have shown that outwardly rectifying Cl^- channels (ORCCs) cannot be found in the apical membranes of confluent Calu-3 monolayers, but are detected when cells are dispersed such that they are no longer polarized. This suggests, but does not prove, that the ORCCs are localized to the basolateral membrane in polarized monolayers. It is possible that this channel is simply inactivated by polarization. More direct proof of basolateral ORCC localization came from similar patch clamp studies of mouse colonic crypt cells which retain polarization for a few hours after dispersion (39). Other studies by Hwang *et al.* (30), used apical membrane permeabilization to show that the basolateral membrane of rat airway epithelium contains a DIDS-sensitive outwardly rectifying Cl^- conductance. However, these investigators showed that basolateral DIDS application inhibits I_{sc}, a result inconsistent with the inhibition of a basolateral anion channel and possibly due to inhibition of another electrogenic process. In patch clamp studies, Uyekubo *et al.* (57) identified an inwardly rectifying Cl^- channel in the basolateral membrane of bovine tracheal epithelium. These investigators proposed a role for this channel in transepithelial Cl^- absorption consistent with the hypotonic ASL model, but did not perform any experiments to verify its involvement in vectorial ion transport. Our studies serve to confirm the existence of basolateral anion channels in human airway epithelium, and further provide strong evidence that these are cAMP-dependent ORCCs.

Having established the existence of basolateral anion channels in airway epithelia, it became necessary to characterize their role in vectorial ion transport. Based on our I_{sc} measurements, we have incorporated basolateral anion channels into the isotonic ASL model of ion transport in airway epithelium depicted in Figure 5.1A. In this model

basolateral NKCC and NBC transport Cl^- and HCO_3^-, respectively, into the cell using the Na^+ gradient produced by the Na^+/K^+-ATPase; HCO_3^- can also be produced inside the cell via the carbonic anhydrase dependent reaction. Stimulation with adenosine, or other cAMP elevating agents, activates apical CFTR and basolateral ORCC. Because the ORCC is relatively more permeable to Cl^- than CFTR, this anion is recycled across the basolateral membrane. In contrast, the greater relative permeability of CFTR for HCO_3^-, over ORCC, results in secretion of this anion across the apical membrane. Thus, the presence of the basolateral ORCC promotes HCO_3^- secretion.

It is becoming progressively clearer that the airway epithelium is not homogenous with regard to the ability to secrete HCO_3^-. Indeed, our studies and those of others show that preferential HCO_3^- secretion can be stimulated by cAMP-dependent mechanisms in Calu-3 but not NHBE cells (13,14,35,55). Interestingly, in our studies the effect of inhibiting basolateral anion channels in Calu-3 cells (29.7 ± 1.1 $\mu A/cm^2$), was almost an order of magnitude greater then its effect in NHBE cells (4.2 ± 0.4 $\mu A/cm^2$). Thus, Calu-3 cells, which preferentially secrete HCO_3^-, express a greater degree of basolateral anion channel activity then NHBE cells. This data suggests that the expression of basolateral anion channels is correlated with the ability to secrete HCO_3^-. This possibility will need to be verified in other model systems. However, it does suggest a mechanism by which some airway epithelia can secrete HCO_3^- preferentially, while others cannot.

Our studies have been carried out primarily under short-circuit conditions and therefore are amenable to interpretation by the isotonic ASL model of airway epithelial ion transport, as described above. However, these findings also have important repercussions for the hypotonic ASL model or airway epithelial ion transport, depicted in

Figure 5.1B. According to this model transepithelial Na^+-absorption occurring via apical ENaC and the basolateral Na^+/K^+-ATPase creates an electrochemical gradient that favours transepithelial Cl^- absorption. However, while the apical mechanism of entry (CFTR) is well established, it was unclear as how the Cl^- gets across the basolateral membrane. Our studies show that basolateral ORCCs are activated in parallel with CFTR by cAMP-dependent mechanisms, thereby providing a transepithelial route for Cl^- absorption. However, transepithelial V_T measurements under open-circuit conditions will be needed to confirm this. If the hypotonic ASL model of ion transport is correct, it would be expected that mutations in the basolateral ORCC should result in a phenotype similar to that of CF. However, it appears that almost all cases of CF can be attributed to mutations in CFTR This can be explained if: 1) ORCC mutations cause severe developmental problems and spontaneous abortion; 2) multiple redundant genes encode a family of ORCCs, which are unlikely to be all mutated at the same time; 3) alternative means of basolateral Cl^- exit, such as anion exchangers, can compensate for the lack of ORCC; 4) the ORCC is actually CFTR acting in an alternative manner; or 5) the hypotonic ASL model of ion transport is incorrect. Thus, the molecular identification of this channel may be a key factor in determining which model of ASL transport is correct, and would further our understanding of the pathogenesis of CF.

To date, the molecular identity of the ORCC remains unknown. Three chloride channel families, whose molecular structures are known, are expressed in the airway epithelium and have all been suggested as the potential source of native ORCC (see chapter 1, section 2.1). The most intriguing possibility, that the ORCC is actually CFTR acting in an alternative manner, perhaps due to alternative splicing or formation of a

multi-subunit complex, is highly unlikely. This was persuasively disproven by Gabriel *et al.* (21) who showed that CFTR null mice (-/-) still display ORCC channels, with unaltered biophysical properties. The CLCA family is the newest to be described and thus the least understood. Therefore, it is possible that some members are cAMP-dependent, or that they become cAMP dependent upon association with an as yet unidentified subunit, leading to the expression of an ORCC like channel. Currently, the most likely candidate family for native ORCC is the voltage-dependent ClCs. Native ORCCs are clearly voltage dependent, displaying outward-rectification and increased activity at depolarizing potentials (30,62). Furthermore, a novel ClC-3 splice variant, ClC-3B, displays properties very similar to that of native ORCC: outward rectification, activation by depolarizing potentials, large conductance, expression primarily in epithelial cells, inhibition by extracellular DIDS, and activation by cAMP (47). Furthermore, it has been shown that ClC-3B contains a PDZ binding motif and suggested that this motif is used to interact with proteins containing PDZ domains that then interact with AKAPs (see chapter 1, section 2.1) (47). We have shown that AKAP inhibitors reduce basolateral DIDS stimulated current in Calu-3 cells, indicating that a similar mechanism may operate for native ORCC. However, it is unlikely that ClC-3B is the ORCC because this channel localizes to the intracellular membranes of native epithelia (23). Thus, the similarities between ClC-3B and the ORCC can only suggest that these channels may be closely related family members. Of course it is entirely possible that the ORCC belongs to an as yet unidentified family of anion channels.

CONCLUSIONS

We have investigated adenosine regulation of airway epithelial ion transport. Our results demonstrate that adenosine receptor distribution is polarized, and that expression of the A_1 receptor, in particular, depends on the source of the tissue. Adenosine homeostasis is maintained by a system of metabolic enzymes and trafficking pathways that can be modulated pharmacologically in order to induce changes in adenosine-dependent ion transport. In general this mediator appears to stimulate anion secretion, particularly through its effects on Cl^- and K^+ channels. Surprisingly, adenosine also activates a basolateral ORCC channel that opposes anion secretion. This channel appears to be involved in determining which anion, Cl^- or HCO_3^-, is secreted. Our findings suggest several therapeutic targets, which may be applicable to the treatment of CF, once we have a better understanding of the pathogenesis of this illness. In this regard, the characterization of normal ion transport and its regulation by the physiological mediator – adenosine, is also beneficial in that it brings us closer to understanding this tragic disease.

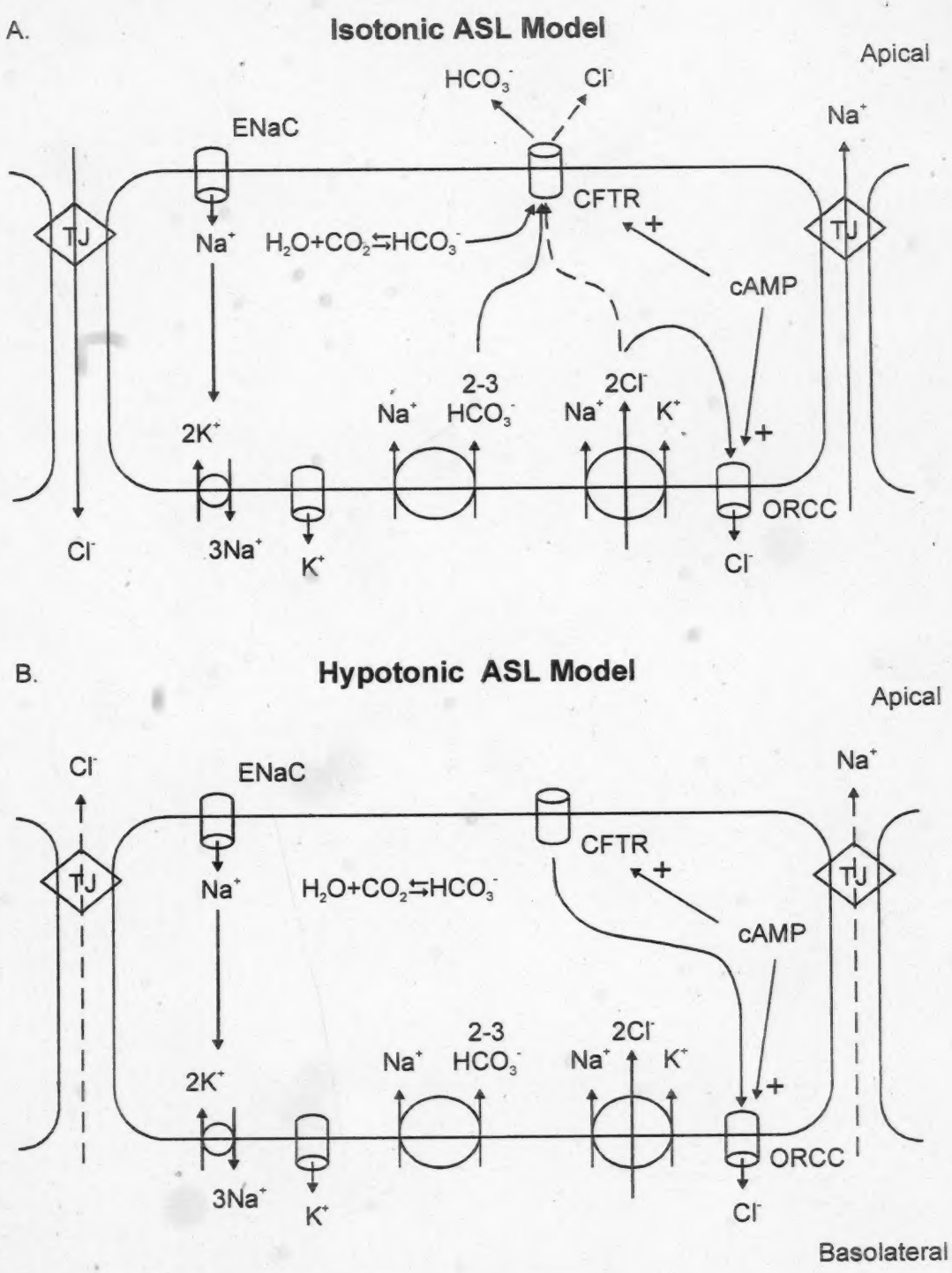
A.
Isotonic ASL Model
Apical
HCO3-
Cl-
ENaC
Na+
CFTR
+
TJ
Na+
H2O+CO2⇆HCO3-
TJ
cAMP
2-3
HCO3-
2Cl-
Na+
Na+
K+
2K+
+
ORCC
Cl-
3Na+
K+
Cl-
B.
Hypotonic ASL Model
Apical
Cl-
ENaC
Na+
CFTR
+
TJ
Na+
H2O+CO2⇆HCO3-
TJ
cAMP
2-3
HCO3-
2Cl-
Na+
Na+
K+
2K+
+
ORCC
3Na+
K+
Cl-
Basolateral

Figure 5.1

FIGURE LEGENDS

Figure 5.1

Basolateral ORCC involvement in the isotonic and hypotonic ASL models of airway epithelial ion transport. A single cell representative of an entire epithelial monolayer is shown schematically with the apical membrane at the top, the basolateral membrane at the bottom and tight junctions (TJ) connecting adjacent cells. Full arrows indicate the major pathways of ion transport; dashed arrows indicate limited, or insignificant pathways. ***A:*** The isotonic ASL model proposes that secretion occurs when Cl^- and HCO_3^- are transported into the cell via basolateral Na^+-dependent cotransporters, with an additional pathway of HCO_3^- production inside the cell, and exit across the apical membrane via CFTR channels that can be activated by cAMP. When basolateral ORCC channels are expressed and activated by cAMP, Cl^- is redirected to the basolateral membrane where it is recycled, thereby making apical CFTR channels available for HCO_3^- conduction. Na^+ will pass through the tight junctions in order to balance the charge separation created. At the same time transcellular absorption of Na^+ occurs via apical ENaC channels and the basolateral Na^+/K^+-ATPase. Cl^- will pass through the tight junctions in order to balance the charge separation created. Basolateral K^+ channels determine membrane potential and thus the magnitude of secretion/absorption, and also influence the type of anion (Cl^- or HCO_3^-) secreted. Net salt secretion will result if transcellular anion secretion dominates over transcellular Na^+ absorption, resulting in an osmotic gradient favouring fluid secretion. Transepithelial water flux (not shown) can occur via paracellular or transcellular (Aquaporin-mediated) pathways. ***B:*** The hypotonic

ASL model proposes that ENaC and the Na^+/K^+-ATPase are responsible for Na^+-absorption, which creates an electrochemical gradient favoring transcellular Cl^- absorption through apical cAMP-dependent CFTR channels and basolateral ORCC channels. In this model, ion movements across the tight junctions are limited, thus the ASL becomes hypotonic.

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